THE EFFECT OF PROTEIN DEFICIENCY AND OTHER DIETARY FACTORS ON THE X ZONE OF THE MOUSE ADRENAL

EVELYN HOWARD AND RICHARD S. BENUA[1]

Department of Physiology, Johns Hopkins University School of Medicine, Baltimore, Maryland

FOUR FIGURES

(Received for publication June 13, 1950)

The X zone of the mouse adrenal is a transitory differentiation of the cortex which has been described in various studies (Howard, '27; Deanesley, '28; Whitehead, '33; McPhail and Read, '42a, b; Jones, '49a, b; and others). It is present in the young of both sexes; in intact males it is not large and disappears at puberty, while in females it develops to a larger size and disappears after an interval of time characteristic of the strain. It hypertrophies after castration in males, but has been shown not to produce physiologically appreciable amounts of androgen (Howard, '46). It has been suggested (Howard, '27) that the X zone might be comparable to the inner part of the human fetal cortex, primarily because its degeneration constitutes a spontaneous regression of the inner portion of the cortex similar to the post natal degeneration of the human fetal reticularis. However, application of the Ashbel and Seligman ('49) histochemical test for lipoidal carbonyl groups reveals that the human fetal cortex is strongly positive, whereas the mouse X zone is negative (Benua and Howard, '50). Hence there is no evidence of a simple homology between the two adrenal regions. It is of interest that the entire adrenal of the hamster does not

[1] Present address: Mayo Foundation, Rochester, Minn.

Copyright, 1950
The Wistar Institute of Anatomy and Biology
All rights reserved

stain in Sudan (Koneff, Simpson and Evans, '46), in this respect resembling the mouse X zone, but that the hamster adrenal is made Sudan positive by estrogens.

In an attempt to get some lead as to the metabolic role of the X zone, we have explored its response to various types of diets. We find that it is markedly suppressed by a partial protein deficiency, is developed to a maximum at moderate levels of dietary protein, and is somewhat reduced

TABLE 1

Composition of diets

(Grams of ingredients to make 100 gm of diet)

INGREDIENTS	DIET SYMBOLS[1]						
	P D	M H	M E	L P	H P	B H	B F
Salt mixture[2]	4	4	4	3	3	4	4
Brewers' yeast powder, Meads	8	8	8	8	8	8	8
Wheat germ oil, Lilly	1	1	1	1	1	1	1
Cod liver oil, Meads	1	1	1	1	1	1	1
Cellu flour	9	9	9	10	10	9	9
Corn oil (Mazola)	5	5	5	..	..	..	22
Casein, crude	4	50	3.5	12	77	25	25
Casein, "vitamin test"	.	..	46.5	..	..	..	..
Corn dextrin	10	10	10	26	..	10	10
Starch	58	12	12	39	..	42	20

[1] Diet symbols: P D, protein deficient; M H, moderately high casein; M E, moderately high alcohol extracted casein; L P, low protein; H P, high protein; B H, low fat; B F, moderately high fat.

[2] Osborne and Mendel (J. Biol. Chem., *34:* 131, 1918).

by extremely high dietary protein. Studies have also been made of the effect of variations in the fat content of the diet and of the influence of feeding purified casein. A few observations have been recorded on the effect of supplementing a protein-deficient diet with methionine, cystine or choline.

EXPERIMENTAL

The composition of the chief diets used is given in table 1. The H strain mice were bred in the laboratory and main-

tained on a commercial animal food.[2] At 17 days of age litters were placed on a preliminary diet similar to the protein-deficient diet described in table 1 except that the casein was increased to 28% and the starch reduced to 34%.

The mice were weaned at 19 to 21 days of age, when the young females were placed on the experimental diets, litter mates being paired for body weight to within 0.5 gm. All diets were fed ad libitum. Males were castrated at 20 to 21 days of age under ether anesthesia and maintained on the preliminary diet until they were 24 to 25 days of age, when litter mates were paired for body weight and placed on the experimental diets. At the end of a 2- to 4-week period the animals were killed and the adrenals carefully cleaned and weighed by the technique previously discussed (Benua and Howard, '45) and prepared for histological study. In one series unilateral adrenalectomy was performed, as is indicated in table 3, under ether anesthesia before the animals were placed on the experimental diets.

The size of the X zone was evaluated in histological sections in a semiquantitative manner by estimating its width relative to the width of the permanent cortex. It was graded on the basis of small, medium or large; i.e., by the numerals 1, 2 and 3, 3 representing a width equal to that of the permanent cortex. The various grades were divided into quarters. Several sections through the region of maximum diameter were evaluated. An evaluation was made taking the glands at random, without knowledge of the treatment the mouse had received. Subsequently a separate evaluation was made in which pairs were specifically compared. Of 66 individuals so graded, the numerical score of 80% of the glands differed only by 0.25 or less.

Measurements of the actual amount of X zone in the glands of 16 mice were made by measuring the areas of the X zone in serial sections in the manner previously used for measurements of cortex and medulla (Benua and Howard, '45). It was found that there was a very satisfactory correlation

[2] Purina fox chow.

between the semiquantitative X zone ratings and the total volume of the X zone actually measured, expressed as the volume: body weight ratio. Since the individual variation in the amount of X zone is great, it was deemed preferable to use the semiquantitative ratings on a larger number of mice, rather than the somewhat more accurate but highly laborious total-volume measurements.

The difference between zones graded 1 or over and zones graded 0.5 or under is qualitative as well as quantitative, in that the very small zones have cells with little cytoplasm whereas the zones graded 1 or over have cells with more abundant cytoplasm, which stains less intensely in hematoxylin. Since the very small zones often appear to be undergoing compression by the surrounding tissue, the functional difference between large and small zones is probably not a simple function of the numerical score.

Protein deficiency

The effects of the protein-deficient diet are shown in table 2. It will be noted that in each of the three series there was a marked decrease in body weight gain in the protein-deficient groups in comparison with the animals on 50% casein. In series I the experiments were run in a different year and hence this series is not directly comparable to the later series. The animals in series II and III were, in general, siblings and differed only in that the castrated males were 4 or 5 days older than the females, which may account for their somewhat reduced rate of growth on both diets. One individual died on the protein-deficient diet after an intestinal infection of several days' duration; otherwise, the deficient groups appeared to be in good general condition and normally active.

The adrenals of the protein-deficient animals were consistently smaller than the glands of the animals on 50% protein, but the adrenal-terminal body weight ratio showed no consistent difference in the three series. The adrenal weight-

initial body weight ratio showed large differences, which were in most cases proportional to the X zone development.

There was a very marked suppression of the X zone by the low protein diet. In the group on 50% casein there were large and well-developed X zones (fig. 3), whereas the litter mates on 4% casein showed only a narrow juxta-medullary border of small darkly staining X zone cells (fig. 4). The

TABLE 2

Effects of protein deficiencies and supplements on growth and adrenal development

SERIES	DIET	NO. OF MICE	GAIN IN BODY WEIGHT	RATIO OF ADRENAL WEIGHT TO		X ZONE SIZE IN ARBITRARY UNITS
				Terminal body weight $\times 10^4$	Initial body weight $\times 10^4$	
			%			
I Females	Protein-deficient, P D [1]	11	42	2.56	3.33	0.5
	50% crude casein, M H [1]	11	77	2.61	4.61	2.3
II Females	Protein-deficient, P D	19	23	2.52	3.04	0.4
	50% crude casein, M H	4	98	2.18	4.30	1.9
	Purified casein, M E	6	96	2.09	4.09	1.2
	P D [2] + 0.5% methionine	8	53	2.00	3.04	0.8
	P D [2] + 0.5% cystine	5	65	2.05	3.38	0.8
	P D [2] + 0.5% choline	6	15	2.62	3.02	0.3
III Castrated males	Protein-deficient, P D	14	6	2.37	2.52	0.5
	50% crude casein, M H	5	73	2.16	3.73	1.9
	Purified casein, M E	7	77	1.88	3.31	1.4
	P D [2] + 0.5% methionine	5	43	1.76	2.51	0.9

[1] In series I the diets contained 3% more Mazola than was given in table 1, replacing an equivalent weight of starch.

[2] Crude casein 3.5%.

Series I, 17 days on diets; series II and III, 14 to 15 days on diets.

adrenals of the low protein group thus contained X zone cells only to the approximate extent to which they are normally present at 21 to 23 days of age, indicating that X zone development had not taken place. The X zone cells in the protein-deficient group frequently appeared compressed, in marked contrast to the larger cells of the well-developed X zones from the 50% casein group.

In series II and III the moderately high casein diet was modified by substituting 46.5% of "vitamin test" casein [3] for an equal amount of crude casein (table 1, ME). This casein is stated by the manufacturers to have undergone several extractions with hot alcohol to remove fats and water-soluble vitamins. Body growth showed no significant alteration but some reduction in the size of the X zone was noted. However, since the number of mice used was not large, this difference between the crude and purified casein must be regarded as suggestive but not definitive.

The X zones in ME mice were invariably better developed than those of the protein-deficient sibling pairs, so that the purified casein did produce a definite improvement in the adrenal condition over that resulting from the protein-deficient diet. The purified casein effect was compared with that of 0.5% of methionine, cystine and choline, each replacing 0.5% of crude casein in the protein-deficient diet. Choline produced no improvement in body growth or adrenal condition. Methionine and cystine produced some improvement in body growth, but not as much as did the purified casein, and very much less improvement in the adrenal. From the data of Block and Bolling ('45) 46.5 gm of casein would be expected to furnish 1.8 gm of methionine plus cystine. Although maximum adrenal stimulation probably is obtained at a lower protein level than 50%, further experiments are needed to determine whether the adrenal effect is due to factors other than the deficiency of the sulfur-containing amino acids.

High protein

In a further experiment the effect of 77% crude casein (HP) was compared with that of 12% (LP). The results are presented in table 3. The high protein diet in females produced no statistically significant impairment of body growth, even in the unilaterally adrenalectomized mice, al-

[3] General Biochemicals, Inc.

though a slight reduction in growth is evident. This suggestive change is in the same direction as the statistically significant growth impairment previously described (Benua and Howard, '45) in unilaterally adrenalectomized males on a high protein diet. This sex difference may be related to the fact that the adrenal is larger in females than in males, and hence the unilaterally adrenalectomized female may be less severely handicapped in the situation under consideration.

In figure 1 a diagram is plotted of adrenal: body weight ratios. The abscissae represent the values on the 12% casein diet (B) and the ordinates the values on the 77% casein diet (MHP). Each point thus represents the values for a litter-mate pair. The intact animals of series IV are here combined with the animals in series V. It will be seen that there is a considerable range of values, probably due in part to genetic factors and to seasonal and temperature effects. The line is drawn through equal values of abscissae and ordinates and hence random distribution of values would give points equally distributed on both sides of the line. It can be seen that there is an asymmetry of distribution, indicating a statistically significant tendency for the adrenals to be somewhat larger on the 12% casein (i.e., high carbohydrate) diet. The mean difference between pairs, as given in the table, is small, but it is interesting when considered in relation to the histological finding of a partial suppression of the X zone on the 77% casein diet, suggesting that involution of the X zone on a high protein diet may account for the reduction in adrenal weight.

In figure 2 are plotted the X zone ratings for the pairs of glands from animals on the high protein (MHP) and high carbohydrate (B) diets. It can be seen that there is an asymmetric distribution indicating relatively larger X zones on the 12% casein diet. Qualitative differences in the X zone were also notable. On the high carbohydrate diet the individual X zone cells were frequently larger, moderately stained, and arranged in reticular cords with very large vascular spaces, while on the high protein diet the cells were

frequently smaller, more darkly stained and often apparently pressed closely together, with blood vessels much less evident. Sometimes the X zone development was very minor and not clearly demarcated from the residual zona reticularis, and this occurred more often on the high protein than on the high carbohydrate diet.

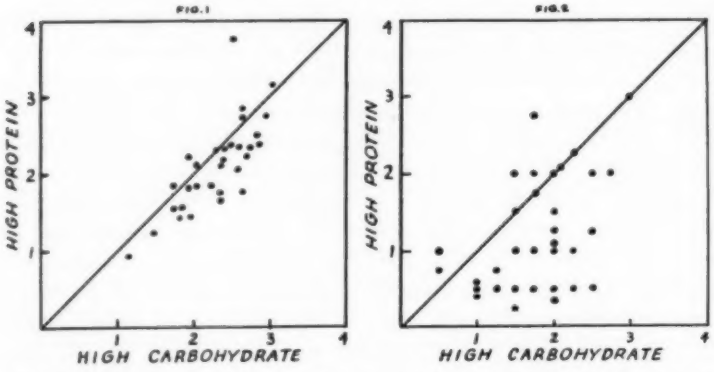


Fig. 1 Adrenal:body weight ratios of paired litter-mate female mice, in which abscissae represent the adrenal:body weight ratios $\times 10^4$ for the members of the pairs on the high carbohydrate diet, LP, and the ordinates the adrenal:body weight ratios for the members of the pairs on the high protein diet, HP. In figures 1 and 2 the line is drawn through equal values of ordinates and abscissae, so that random scatter would give equal distribution on both sides of the line.[4]

Fig. 2 The X zone size for litter-mate pairs of females, in which the abscissae represent the X zone in arbitrary units for the member of the pair on the high carbohydrate diet, LP, and the ordinates the X zone size for the member of the pair on the high protein diet, HP.

[4] We wish to thank Dr. Margaret Merrell for advice regarding the statistical treatment of our data.

Signs of the normal concurrent regression of the X zone were evident in this series. Thus, in three pairs of mice the X zone showed a more advanced state of involution on the high carbohydrate diet than on the high protein and in 9 pairs there was no evident qualitative difference, while in 23 pairs the X zone on the high protein diet showed either more degenerative change or smaller cells. Thus we find

qualitative indications of less favorable conditions for the maintenance of the X zone on the 77% casein diet, as well as a quantitative reduction in the size of the zone. However, the variability of the material is considerable, and the dietary factor can be only one of the many factors affecting the state of the adrenal.

In the unilaterally adrenalectomized animals the amounts of X zone were quite small on both diets, and the gland weights showed no significant effect of the high protein diet. This is interpreted to indicate that unilateral adrenalectomy tends to suppress X zone development to such an extent that any further suppression by the high protein diet is not appreciable.

Unilateral adrenalectomy was shown by Whitehead ('33) to be followed by a temporary drop in sudanophil cortical lipoid in male mice, and by a decrease in the intensity of the Schultz cholesterol reaction in the cortex of guinea pigs (Simmons and Whitehead, '37). Control laparotomies produced such changes much more rarely. In view of the findings of Long ('47) that adrenal cholesterol is reduced in various types of stress, one may infer that unilateral adrenalectomy produces a significant degree of relative stress on the remaining adrenal. The X zone, then, appears to be a differentiation which the cortex tends to discard under this type of stress.

Evidence of another dietary factor affecting the X zone was obtained in comparing the adrenals of female mice on the Benua-Howard ('45) BS diet with those on chow. As is shown in table 3, there was no marked difference in the adrenal: body weight ratio, but there was a reduction in the amount of X zone in the mice on the chow diet. The specific composition of BS diet is given elsewhere (Benua and Howard, '45); its percentage composition was protein 24, fat 19 and carbohydrate 33. Chow is specified by the manufacturer to contain over 21% protein.

The BS diet thus appears to be more favorable than chow for the development of X zone and also appreciably better

for body growth. Since chow is made of natural foodstuffs, and since androgens are highly effective in suppressing the X zone (Howard, '40), it was decided to determine whether chow contained appreciably more androgen than the synthetic foods. In a study reported elsewhere (Benua and Howard, '46) it was found that chow had no appreciable effect on the prostates of castrated rats. Accordingly, it appears that

TABLE 3

The influence of high protein and moderately high fat diets on the adrenal

SERIES	DIET [1]	NO. OF ANIMALS	TIME ON DIET	INITIAL BODY WT.	BODY WT. GAIN	ADRENAL WT.-BODY WT. RATIO $\times 10^4$	X ZONE
			days	*gm*	%		*arb. units*
IV	L P	13	20–21	9.6	95	2.29	1.6
	M H P	13	20–21	9.6	95	1.99	1.1
	L P [2]	12	20–21	9.9	90	1.18	0.9
	M H P [2]	12	20–21	9.9	88	1.18	1.0
V	L P	17	18–23	10.4	88	2.34	1.9
	H P	17	18–23	10.6	79	2.22	1.3
VI	Chow	10	28	11.7	74	2.57	1.3
	B S	10	28	11.4	94	2.46	1.7
VII	B H	18	28	...	..	2.44	1.9
	B F	18	28	...	..	2.33	1.4
VIII	B H	22	18	10.8	89	2.25	1.5
	B F	22	18	10.7	93	2.41	1.6

[1] See footnote 1, table 1.
[2] Left adrenal removed.

the synthetic diet was favorable to the development of the X zone for some unknown reason.

A study was also made of the effects of replacing part of the dietary carbohydrate with a mixed fat. The composition of the high and low fat diets is given in table 1 and the results are presented in table 3. It may be seen that in series VII there was some reduction in X zone size on the high fat diet. In this group the fur became oily so that the hairs stuck together, a circumstance which must have con-

siderably reduced the heat insulating capacity of the fur, so that these mice were subjected to a higher rate of heat loss than were their paired controls. During these experiments the mouse room temperatures were 16 to 22°C. In series VIII feeding arrangements were modified to minimize fur soiling, and the figures show no tendency for the X zone to be reduced on the high fat diet. Body growth and adrenal: body weight ratios were, on the contrary, slightly greater on the high fat, but the differences were not highly significant. There was no gross difference in the amount of adipose tissue present. Ingle et al. ('43) also observed slightly heavier adrenals in rats on a high fat diet. Histological study of the glands of the animals in series VIII revealed no difference in the X zones which could be related to the diets. The X zones were of medium size and showed various degrees of the degenerative involution normal for this age group. Hence, moderately high dietary fat may be excluded as a factor influencing X zone growth or degeneration under the conditions of these experiments.

DISCUSSION

An adrenal hypertrophy of 40 to 50% on high protein diets in rats has been observed by Tepperman, Engel and Long ('43), while Ingle ('45) and Leathem ('47) found somewhat smaller increases. Benua and Howard ('45) failed to observe adrenal hypertrophy in male mice when comparing animals on a 77% casein with those on a 12% casein diet. In female mice, on the contrary, the adrenals have now been found to be actually smaller after the animals have been on a 77% casein diet than when they have been fed only 12% casein. Nevertheless, in comparing the 50% casein diet with the 4%, we do find qualitative and quantitative evidence of adrenal stimulation at the higher casein level. It is possible that the factors producing adrenal enlargement in the rat on a high casein diet are operating in this lower range of dietary protein in the mouse. Mills ('44) has noted that protein requirements for rapid growth (expressed as per

cent of the diet) are increased with a rise in environmental temperature. Consequently, the per cent of protein in the diet required to show a given physiological effect may be expected to be influenced by temperature, and probably by other factors.

In accordance with Mills' ('44) observation of a need for a higher percentage of casein in the diet to produce maximum growth at high temperatures than at moderate temperatures, it might be expected that our 12% casein diet would be suboptimum in hot weather. Our data do indeed show that, while 26 females on 12% casein which were studied between December and April had an average X zone rating of 1.9, 8 females on 12% casein studied during June in Baltimore had consistently small X zones, with an average rating of only 0.9. While no direct tests of the point have been made, the data suggest that possible effects of environmental temperature should be borne in mind in making studies of the X zone.

Adrenalectomized animals are able to metabolize dietary protein (Ingle, Ginther and Nezamis, '43). Engel ('49) finds that the protein catabolic response to adrenal cortical extract depends on "the internal metabolic environment of the organism at the time of treatment," the need for new carbohydrate being an important factor determining the occurrence and magnitude of the protein catabolic response. Since in the present experiments with diets fed ad libitum there was presumably a minimum need for tissue protein catabolism, and furthermore since the adrenocorticotrophic hormone (ACTH) does not exert a trophic effect on the X zone (Jones, '48, '49b), one need not infer that the formation of the X zone on the 12 and 50% casein diets was related to the protein catabolic role of adrenal steroids. The observation that growth is significantly retarded after unilateral adrenalectomy on high protein diets in males (Benua and Howard, '45) does suggest that the adrenal is involved in some way in the metabolism of dietary protein,

if only by promoting the well-being of the kidney. There is increased blood non-protein nitrogen in rats on high protein diets (Leathem, '47).

The X zone reduction on very high levels of dietary protein may not necessarily be related to its suppression on low levels. It has been shown by Addis et al. ('40) that there is maximum retention of protein at a moderate dietary level, varying somewhat according to circumstances but being reduced at high levels. The high casein diets used in the present study presumably impose renal and other stresses.

It has been observed that 3.5% protein diets resulted in cessation of estrus in rats (Guilbert and Goss, '32) and that chronic inanition apparently depressed the pituitary output of gonadotrophins (Mulinos and Pomerantz, '40), while Handler and Bernheim ('50) concluded that there was diminished ACTH production by the pituitaries of rats on an 8% casein diet. Jones ('48, '49b) has observed that a pituitary gonadotrophin seems to be a maintenance factor for the X zone in hypophysectomized mice; consequently, the pituitary may be involved in the effect of protein deficiency on the adrenal. However, McPhail and Read ('42b) observed that some pituitary extracts caused X zone involution in intact mice.

The X zone is known to involute rapidly during pregnancy (Howard, '27). McPhail and Read ('42a) have observed that if females are not permitted to suckle their young, regeneration of the X zone may occur following parturition. It would seem possible that the protein drain of pregnancy and lactation (literature cited by Sahyun, '48) may be a factor in the X zone involution in these states. Progesterone and estrogen treatments do not have an effect on the adrenal comparable to that of pregnancy, as is discussed by Howard and Gengradom ('40). Androgens cause a prompt disappearance of the X zone (Howard, '40; Jones, '49a; and others).

It would be interesting to know whether the nitrogen anabolic effect of androgen (literature cited by Sahyun, '48) is related to this adrenal response.

The finding that the X zone is suppressed in protein deficiency raises the question of whether this may reflect a specific relationship of the adrenal to protein metabolism, or whether it is a relatively non-specific response to a situation in which a number of cellular processes in the growing animal must find themselves limited by a relative shortage of building units. If the latter should be the case, it would seem that the X zone is more vulnerable than the adrenal as a whole.

SUMMARY

1. A partially protein deficient diet caused a very marked suppression of the X zone of the mouse adrenal.

2. A 77% casein diet resulted in appreciable reduction of the X zone, indicating that the X zone is maximally developed at moderate levels of dietary protein.

3. Replacement of a part of the carbohydrate by a mixed fat was without any marked effect on the X zone.

4. The X zone was larger on a synthetic diet than on chow.

LITERATURE CITED

ADDIS, T., D. D. LEE, W. LEW AND L. J. POO 1940 The protein content of the organs and tissues at different levels of protein consumption. J. Nutrition, *19:* 199.

ASHBEL, R., AND A. M. SELIGMAN 1949 A new reagent for the histochemical demonstration of active carbonyl groups. A new method for staining ketonic steroids. Endocrinology, *44:* 565.

BENUA, R. S., AND E. HOWARD 1945 The effect of dietary protein on adrenal weights and on growth after unilateral adrenalectomy. Ibid., *36:* 170.

———— 1946 A note on the absence of androgen from a diet of natural foods. Ibid., *38:* 260.

———— 1950 A carbonyl reaction differentiating the fetal zona reticularis of the human adrenal cortex from the mouse X zone. Bull. Johns Hopkins Hosp., *86:* 200.

BLOCK, R. J., AND D. BOLLING 1945 The Amino Acid Composition of Proteins and Foods. Charles C. Thomas, Springfield, Ill.

DEANESLY, R. 1928 A study of the adrenal cortex in the mouse and its relation to the gonads. Proc. R. Soc., London, Series B, *103:* 523.

ENGEL, F. L. 1949 Studies on the nature of the protein catabolic response to adrenal cortical extract. Accentuation by insulin hypoglycemia. Endocrinology, *45:* 170.

GUILBERT, H. R., AND H. GOSS 1932 Some effects of restricted protein intake on the estrous cycle and gestation in the rat. J. Nutrition, *5:* 251.

HANDLER, P., AND F. BERNHEIM 1950 Diminished ACTH production in rats on low protein diets: explanation of relationship between dietary protein and renal hypertension. Fed. Proc., *9:* 399.

HOWARD, E. 1927 A transitory zone in the adrenal cortex which shows age and sex relationships. Am. J. Anat., *40:* 251.

———— 1940 Regarding the effects of desoxy-corticosterone and of testosterone on the adrenal X zone. Anat. Rec., *77:* 181.

———— 1946 The effect of adrenalectomy on the accessory reproductive glands of mice castrated for short periods. Endocrinology, *38:* 156.

HOWARD, E., AND SAIYUDE GENGRADOM 1940 The effects of ovariectomy and administration of progesterone on the adrenal X zone and the uterus. Ibid., *26:* 1048.

INGLE, D. J. 1945 A further study of the effect of diet on adrenal weights in rats. Ibid., *37:* 7.

INGLE, D. J., G. B. GINTHER AND J. NEZAMIS 1943 Effect of diet in rats on adrenal weights and on survival following adrenalectomy. Ibid., *32:* 410.

JONES, I. C. 1948 Relationship of the pituitary to the X zone of the mouse adrenal. Proc. Soc. Exp. Biol. Med., *69:* 120.

———— 1949a The action of the testosterone on the adrenal cortex of the hypophysectomized, prepuberally castrated male mouse. Endocrinology, *44:* 427.

———— 1949b The relationship of the mouse adrenal cortex to the pituitary. Ibid., *45:* 514.

KONEFF, A. A., M. E. SIMPSON AND H. M. EVANS 1946 Effects of chronic administration of diethylstilbestrol on the pituitary and other endocrine organs of hamsters. Anat. Rec., *94:* 169.

LEATHEM, J. H. 1947 Plasma protein concentrations and organ weights of rats as related to a high protein diet. Proc. Soc. Exp. Biol. Med., *64:* 90.

LONG, C. N. H. 1947 The relation of cholesterol and ascorbic acid to the secretion of the adrenal cortex. Recent Progress in Hormone Research. Proc. Laurentian Hormone Conference, *1:* 99.

MCPHAIL, M. K., AND H. C. READ 1942a The mouse adrenal. I. Development, degeneration and regeneration of the X zone. Anat. Rec., *84:* 51.

———— 1942b The mouse adrenal. II. The action of certain hormonal substances on the adrenal gland of the mouse, with particular reference to their action on the X zone. Ibid., *84:* 75.

MILLS, C. A. 1944 Environmental temperature and protein requirement. Arch. Biochem., *5:* 333.

MULINOS, M. G., AND L. POMERANTZ 1940 Pseudo-hypophysectomy: a condition resembling hypophysectomy produced by malnutrition. J. Nutrition, *19:* 493.

SAHYUN, M. 1948 Proteins and Amino Acids in Nutrition. Reinhold Publishing Corp., New York, N. Y.

SIMMONS, H. T., AND RAYMOND WHITEHEAD 1937 Fatty changes in the adrenal cortex of guinea-pigs after unilateral adrenalectomy. J. Pathol. Bacteriol., *45:* 441.

TEPPERMAN, J., F. L. ENGEL AND C. N. H. LONG 1943 Effect of high protein diets on size and activity of the adrenal cortex in the albino rat. Endocrinology, *32:* 403.

WHITEHEAD, R. 1933 Growth and mitosis in the mouse suprarenal. J. Anat., *67:* 399.

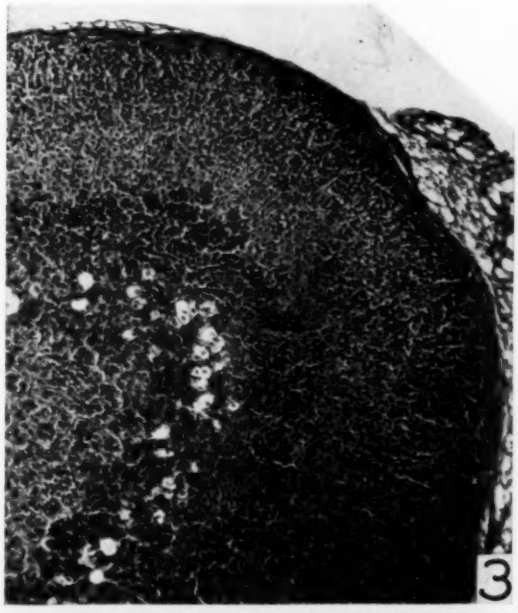
PLATE 1

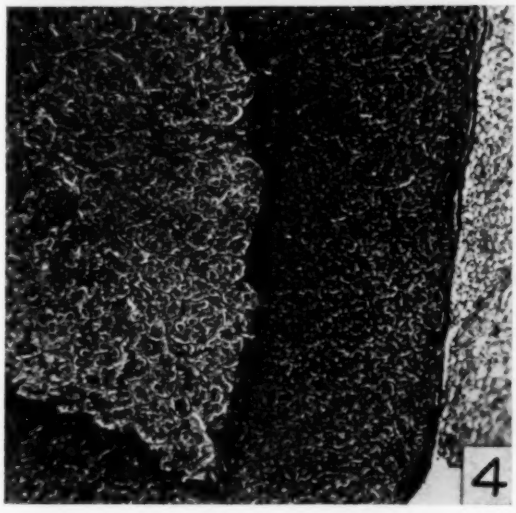
EXPLANATION OF FIGURES

3 and 4 Sections from adrenals of two litter-mate castrated male mice at 37 days of age, after 14 days on the experimental diets. Hematoxylin and eosin stain. × 100.

3 Adrenal of mouse on 50% crude casein diet, MH, showing well-developed X zone with beginning involution.

4 Adrenal of mouse on protein-deficient diet, PD, showing suppression of X zone.


3


4

THE FECAL EXCRETION OF LIPIDS BY RATS AS INFLUENCED BY THE DIET [1]

J. A. SCHULZ AND BYRON H. THOMAS
Chemistry Section, Iowa Agricultural Experiment Station, Ames

(Received for publication November 21, 1949)

There has not, apparently, been a great deal of research conducted on the effect of various adjuvants to the diet of rats upon the digestibility and excretion of fats. Boyd, Crim and Lyman ('32), who reported upon the digestion and absorption of calcium soaps by rats, presented a good bibliography of earlier work concerned with the effect of calcium salts upon fat absorption. Steenbock and associates ('35) found that bile, sodium benzoate, alcohol, peptone and cane sugar decreased fat absorption in rats; and Frazer ('46) reported the effect of choline on the intestinal absorption of fat. Barnes, Primrose and Burr ('44) found that while the absorption of a fat such as steam lard was influenced only slightly, lard that contained hydrogenated fat was digested more completely when the protein of the diet approximated 30% than when only a 14% protein diet was fed.

Deuel and associates (Deuel and Hallman, '40a; Deuel et al., '40b; Augur et al., '47; Crockett and Deuel, '47), Hoagland and Snider ('40, '43), Mattil ('46), Burr and Barnes ('43b), and Wikoff, Koonce and McGuire ('43) have reported extensively on the digestion by rats of artificial, natural and hydrogenated fats, whereas Deuel and Hallman ('40a) and Mattil and Higgins ('45) have studied the digestibility of certain artificial triglycerides.

[1] Journal paper no. J1721 of the Iowa Agricultural Experiment Station. Project no. 507.

Levin and Smith ('27) have reported that rats were able to utilize 98 and 99% of the fat in their diets, as measured by the ether extract of their feces, when the diets contained from 22 to 64% lard, and the work of Bloor ('38, '39), Burr and Barnes ('43a) and Eckstein ('41) has apparently established the fact that the normal fecal lipid excretion of rats, like that of most other species, while influenced by the amount and kind of fat in the diet, is not entirely dependent upon it.

Numerous researchers have studied the effect of lactose in the diets of rats, but these studies have been primarily concerned with the relative nutritional value of the sugars, the production of cataracts, the effect of a change in the hydrogen ion concentration of the intestinal tract on the absorption of calcium and phosphorus, or the effects of lactose on gastrointestinal motility, without specific reference to the lipids. These latter studies will be considered later. There have also been several studies on the effect of crude fiber in the diet upon gastrointestinal motility and the excretion of certain nutrients, but a specific study of lipid excretion does not seem to have to been an objective.

A few years ago the present authors reported the effects of various adjuvants to the diet of rats on the changes in body fats induced by the feeding of soybean oil (Schulz and Thomas, '45). The feces obtained from the rats during these experiments were collected daily and stored in metal containers. The present article is a report of the effect these various diet adjuvants had upon the excretion by the rats of fatty acids and non-saponifiable lipids.

EXPERIMENTAL

The study consisted of two experiments. In the first experiment 13 lots of 6 weanling rats each (three males and three females), averaging 56 gm in weight, each rat individually caged and attended, were fed a diet composed of 50% solvent-processed soybean oil meal, 1.5% salt mixture, 2% yeast or 1% yeast concentrate,[2] 10% soybean oil, cod

[2] Harris; from the Harris Laboratories, Tuckahoe, N. Y.

liver oil concentrate,[3] and cane sugar to make 100%. Lot 2 received this basal ration; lot 1 received no soybean oil; lot 9 received 30% soybean oil; lot 16 received lactose instead of sucrose; and to the basal diet of the other lots certain adjuvants which might have some effect upon fat metabolism were added. The different adjuvants replaced equal weights of sugar where more than 1% of the adjuvant was fed; otherwise the adjuvant was merely added to the basal ration.

In experiment 1 the rats were kept on test until they reached a weight of 250 gm or had ceased growing. Because of the nature of the diets fed, the rats both within and among the different lots grew at rather widely different rates; so that when the experiment was terminated, the number of days the different rats were on test varied considerably. The rats used in experiment 1 were on test an average of 95 ± 4.0 days. Their final weight averaged 205 gm and their average weight gain was 152 gm each. It was necessary for various reasons to discard one rat from each of lots S9, S13 and S16. Therefore there were only 5 rats in each of these lots at the close of the experiment.

In the second experiment 6 lots of 7 weanling rats each, all males averaging 70 gm in weight and managed as indicated above, were fed the same basal diet as those in experiment 1 except that corn starch replaced the cane sugar. These animals grew much more uniformly than did the rats used in experiment 1; they had attained an average body weight of 294 gm, with an average weight gain of 224 gm each, when they were taken off experiment after an average of 77.8 ± 0.3 days. Lot 23 contained only 6 rats at the close of the experiment.

Further details concerning the nature of the experiments and the management of the rats are recorded in Research Bulletin 336 of the Iowa Agricultural Experiment Station (Schulz and Thomas, '45). Table 1 lists the diets fed in these experiments.

[3] Upjohn.

TABLE 1

Adjuvants fed and amount of each which was added to or which replaced sugar or starch in the basal diets

LOT NO.	ADJUVANT AND AMOUNT IN DIET		LOT NO.	ADJUVANT AND AMOUNT IN DIET	
		Experiment 1			
S1	Positive control	No soybean oil	S9	Negative control	30% soybean oil
S2	Basal diet	....	S10	Agar-agar	10%
S3	Charred alfalfa meal	20%	S11	Casein	20%
S4	L-Cystine	0.5%	S12	Carotene (per rat)	0.0140 gm per wk.
S5	DL-Methionine	0.5%	S13	Calcium carbonate	1st mo. 2%, then 1%
S6	Ascorbic acid (per rat)	25 mg per day	S14	Calcium chloride	1st mo. 2.2%, then 1.1%
S7	Bile salts	0.5%	S15	Calcium lactate	1st mo. 6.2% then 3.1%
S8	Sodium fluoride	0.05%	S16	Lactose replaced all sucrose	36.5%
		Experiment 2			*gm per rat per wk.*
S19	Basal diet	....	S22	Carotene	0.0070
S20	Choline chloride (Merck)	1%	S23	Carotene	0.0140
S21	Niacin	1%	S24	Carotene	0.0280

BASAL DIETS [1]	EXPT. NO. I	EXPT. NO. II
Soybean oilmeal (solvent-extracted = 0.5% fat)	50	50
Soybean oil	10.0	10.0
Salt mixture ($CaCO_3$ 55; bone meal 20; NaCl 20; ferric citrate 4.5; KI 0.05% [2])	1.5	1.5
Yeast	2.0	0.0
Yeast concentrate	0.0	1.0
Cane sugar	36.5	0 0
Starch	00.0	3.75
Total	100.0%	100.0%

[1] Each rat received weekly three drops of cod liver oil concentrate.

[2] Lot 14: salt mixture = $CaCl_2$ 55; $Ca_3(PO_4)_2$ 20; NaCl 20; ferric citrate 4.5; KI 0.05%.

All the feces obtained from each rat were collected each day throughout the duration of the experiment, and these composited feces were stored in light-proof tin containers. Previous to analysis each can of feces was ground to pass

TABLE 2

Average feed ingested and feces excreted daily

LOT NO. AND ADJUVANT FED		AVERAGE FEED INGESTED DAILY	AVERAGE LIPIDS INGESTED DAILY	AVERAGE FECES EXCRETED DAILY	% FECES EXCRETED [1]
		gm	*gm*	*gm*	
		Experiment 1			
S1	Low fat-sucrose diet	10.78	0.05	1.20	11.1
S2	Basal sucrose diet	9.52	1.00	1.20	12.7
S3	Charred alfalfa	9.16	1.00	2.71	29.5
S4	L-cystine	9.36	0.98	1.15	12.3
S5	DL-methionine	9.17	0.96	1.08	11.8
S6	Ascorbic acid	9.37	0.98	1.09	11.7
S7	Bile salts	9.47	0.99	1.26	13.4
S8	Sodium fluoride	9.94	1.04	1.20	12.1
S9	30% soybean oil	8.57	2.61	0.97	11.3
S10	Agar-agar	9.90	1.04	2.06	20.8
S11	Casein	11.28	1.19	1.35	12.0
S12	Carotene	9.59	1.08	1.19	12.5
S13	$CaCO_3$	8.81	0.92	1.23	13.9
S14	$CaCl_2$	8.87	0.93	1.07	12.0
S15	Calcium lactate	8.23	0.86	1.03	12.5
S16	Lactose	10.32	1.08	1.74	16.7
Standard error of a mean (6 rats)		± 0.50	± 0.059	± 0.09	± 0.49
Standard error of a mean (5 rats)		± 0.55	± 0.065	± 0.10	± 0.54
		Experiment 2			
S19	Basal starch diet	13.26	1.39	0.79	6.0
S20	Choline	12.47	1.31	0.78	6.3
S21	Niacin	12.79	1.34	0.83	6.5
S22	Carotene	13.06	1.37	0.91	7.0
S23	Carotene	12.24	1.30	0.89	7.3
S24	Carotene	12.36	1.32	0.88	7.1
Standard error of a mean (7 rats)		± 0.41	± 0.043	± 0.03	± 0.13
Standard error of a mean (6 rats)		± 0.44	± 0.046	± 0.03	± 0.14

[1] Weight feces × 100/weight of feed ingested.

a 60-mesh sieve and thoroughly mixed. The fatty acids and non-saponifiable lipids were then determined upon separate 2-gm aliquots of the feces as follows: 2 gm of ground feces, 4 gm of solid KOH, 5 ml water and 35 ml C_2H_5OH (95%) were placed into 250-ml erlenmeyer flasks and these flasks, fitted with cold finger condensers, were placed upon a steam hot plate. After the saponification had continued for 4 hours, the condensers were removed, water was added and the liquids in the flasks were evaporated to small volumes to remove most of the alcohol. The residues were then washed with water into 250-ml separatory funnels and extracted repeatedly with diethyl ether to remove the non-saponifiable lipids. The combined ether extracts were permitted to evaporate to dryness at laboratory temperature; the residues were dissolved in diethyl ether and filtered into 50-ml erlenmeyer flasks which contained anhydrous sodium sulfate. These flasks were then stoppered and placed in a refrigerator. After several hours, the dried ether extracts were filtered from the sodium sulfate, allowed to evaporate to dryness, again taken up in anhydrous diethyl ether and filtered into tared beakers. The residues after evaporation at room temperature were dried to constant weight at 70°C. in a vacuum oven and reported as non-saponifiable lipids.

The fatty acids were determined in the same manner except that the alkaline saponifications of the rat feces were acidfied with 20% HCl before being washed into separatory funnels and extracted with ether. Since the residues obtained in this case consisted of both the fatty acids and the non-saponifiable lipids, the weight of the non-saponifiable lipids, obtained first, was subtracted to obtain the weight of the fatty acids. All determinations were made in duplicate and where results were not satisfactory further replications were made.

RESULTS

Table 2 lists the average amounts of feed ingested and feces excreted daily by the different lots of rats in both experiments 1 and 2. In experiment 1, the basal diet that

contained 10% soybean oil (lot 2) is considered the diet that provided "normal" feces, whereas in experiment 2, in which starch replaced the sucrose in the diet, the rats in lot S19 received the basal starch diet.

If the "weight of the feces excreted" $\times$ 100/"weight of feed ingested" is taken as the percentage of feces excreted, it is found that in experiment 1 the addition of 20% of charred alfalfa or the addition of 10% of agar-agar to the diet, or the replacement of the sucrose in the diet by lactose, resulted in an increased percentage excretion of feces. Statistical analyses of the figures[4] show that since the rats in lot S2 excreted 12.3% feces, 14.7% excretion is the necessary mean to be judged significantly greater than this. The rats in lots S1, S5, S6, and S9 excreted smaller percentages of feces than did the rats in lot 2, but these differences were not significant. Apparently the inclusion of L-cystine, DL-methionine, ascorbic acid, bile salts, sodium fluoride, casein, calcium carbonate, calcium chloride or calcium lactate in the basal diet of the rats used in experiment 1, in the amounts here specified, had no statistically significant influence on the percentage of fecal excretion by these rats. This percentage was also not influenced by the exclusion of the 10% soybean oil in the basal diet nor by the increase of the soybean oil to 30% of the diet.

In experiment 1 the rats which received carotene in their diet (lot S12) did not excrete a percentage of feces significantly different from that of those rats which received the basal sucrose diet (lot S2). In experiment 2 statistical analysis shows that at the $P = 0.01$ level all the lots of rats that received carotene at different levels of the diet (lots S22, S23 and S24) excreted increased percentages of feces when compared with the lot of rats which received the basal starch diet (lot S19).

Since experiments 1 and 2 were conducted at different times, it is not statistically valid to compare them. However,

[4] Grateful acknowledgment is made to Mr. J. G. Darroch of the Statistical Laboratory of the Iowa State College for statistical analyses.

it seems worthy of note that the rats used in experiment 1 excreted approximately twice the percentage of feces excreted by the rats used in experiment 2.

Table 3 lists the figures showing the extent of the excretion and the apparent retention of dietary lipids by the

TABLE 3

Excretion and retention of lipids in the diet

LOT NO.	AVERAGE WT. F.A. IN FECES DAILY [1]	AVERAGE WT. N.S.L. IN FECES DAILY [1]	AVERAGE WT. TOTAL LIPIDS APPARENTLY RETAINED DAILY	PER CENT LIPIDS APPARENTLY RETAINED DAILY
	gm	*gm*	*gm*	%
		Experiment 1		
S1	0.047	0.031	(—) 0.026	
S2	0.072	0.035	0.892	89.3
S3	0.112	0.044	0.838	84.4
S4	0.070	0.032	0.881	89.5
S5	0.068	0.034	0.860	89.5
S6	0.073	0.033	0.873	89.3
S7	0.075	0.034	0.884	89.0
S8	0.072	0.033	0.937	90.0
S9	0.062	0.038	2.514	96.2
S10	0.062	0.038	0.942	90.4
S11	0.077	0.036	1.082	90.6
S12	0.079	0.035	0.963	89.5
S13	0.063	0.030	0.831	90.0
S14	0.070	0.028	0.833	89.5
S15	0.056	0.022	0.787	91.0
S16	0.098	0.031	0.952	88.1
S.E.[2]	(6) ± 0.0075	± 0.0028	± 0.0538	± 0.59
S.E.[2]	(5) ± 0.0082	± 0.0030	± 0.0590	± 0.65
		Experiment 2		
S19	0.046	0.021	1.320	95.1
S20	0.047	0.020	1.238	94.8
S21	0.051	0.021	1.267	94.6
S22	0.059	0.020	1.295	94.2
S23	0.060	0.021	1.214	93.7
S24	0.059	0.019	1.245	94.1
S.E.[2]	(7) ± 0.0031	± 0.0014	± 0.0412	± 0.19
S.E.[2]	(6) ± 0.0033	± 0.0016	± 0.0446	± 0.20

[1] F.A. = fatty acids; N.S.L. = non-saponifiable lipids.

[2] S.E. (5), (6) or (7) = standard error of a mean obtained by using results from 5, 6 or 7 rats.

rats in both experiments 1 and 2. Statistical analysis using $P = 0.01$ shows that in experiment 1 the inclusion of 20% charred alfalfa in the diet (lot 3) significantly decreased the apparent percentage retention of the lipids, and that the substitution of 30% soybean oil in place of the 10% soybean oil contained in the basal ration significantly increased the apparent percentage retention of lipids when the apparent percentage retention is compared with that established by the rats which received the basal ration (lot 2). The rats in lot 1, having received no added oil in their low fat diet, excreted more lipids than they ingested and thus established an apparent negative percentage retention. The inclusion of 10% agar-agar (lot 10) and the substitution of lactose for sucrose in the diet (lot 16), like the inclusion of charred alfalfa, resulted in an increased excretion of feces but had no significant effect upon the percentage of lipids apparently retained by the rats which received these diets.

However, if the figures are calculated for the ratio of fecal excretion of fatty acids to fecal excretion of non-saponifiable lipids (fecal F.A./fecal N.S.L.), it is found that this ratio for the feces produced by the rats that received the basal diet (lot S2) is 2.13 ± 0.25, whereas for the feces produced by the rats that received the lactose diet (lot 16) the ratio is 3.24 ± 0.42. These ratios differ significantly; calculations also show that the inclusion of charred alfalfa in the diet apparently increased this F.A./N.S.L. ratio slightly, while the exclusion of soybean oil, the inclusion of 30% soybean oil and the inclusion of 10% agar-agar, respectively, in the basal diets apparently decreased this F.A./N.S.L. ratio when compared to the ratio established by the rats in lot 2. These latter differences were not, however, large enough to be significant and are cited merely as possible factors which might under certain conditions affect the differential excretion of the fatty acids and the non-saponifiable lipids.

Statistical analysis using $P = 0.01$ shows that in experiment 2 the rats which received the three levels of carotene

in their diets (lots 22, 23, and 24) apparently retained significantly decreased percentages of total lipids when compared with the rats in lot 19, which received the basal starch diet.

DISCUSSION

On the basis of these experiments it appears that the fecal excretion of the rats was increased by the inclusion of charred alfalfa and agar-agar and by the substitution of lactose for sucrose in a sucrose diet. Of the three, however, only the inclusion of charred alfalfa resulted in an alteration of the apparent percentage retention of lipids. The charred alfalfa was found by analysis to contain 29.7% crude fiber, whereas the same analytical method showed only 0.80% crude fiber in the agar-agar.

Many investigators have studied the effects of varying percentages of lactose in the diet of rats upon gastric motility. While the results in some cases were controversial, there seemed to be general agreement that diarrhea of varying degrees of intensity resulted when lactose constituted more than 20% of the diet. A few of these investigators considered the effects of lactose upon digestibility of the organic nutrients in the diet, and concluded that the feeding of lactose resulted in impairment of the digestion of certain organic nutrients and in an increased excretion of food residues. The subject of the effect of lactose upon the gastrointestinal motility of various species has recently been very capably reviewed by Fischer and Sutton ('49).

The rats in lot 16 of the present experiments, which received the lactose diet, all developed a diarrhea of varying degrees of intensity, but this diarrheal condition moderated and then ceased within a period of a week, after which the feces were hard, black and brittle when dry. We agree with those investigators who found that lactose in the diet increased the percentage of feces excreted, but are not prepared to state whether or not this condition is the result of food residues. As is stated above, although the lactose diet increased the ratio of fatty acids to non-saponifiable

lipids excreted, it did not significantly affect the apparent percentage retention of total lipids.

As was noted previously, while it is not statistically valid to compare the results obtained in experiments 1 and 2, it seems desirable to call attention to the fact that, in addition to the differences in fecal excretion, there were also differences that resulted when carotene was fed in the two experiments. In experiment 1 the addition of carotene to the sucrose diet resulted in a decreased iodine number for the body fat of the rats (Schulz and Thomas, '45) but in no alteration in the amount of feces excreted or lipids retained.

In experiment 2 the inclusion of carotene in the basal starch diet at three levels resulted in no change in the iodine numbers of the body fats (Schulz and Thomas, '45), in an increased percentage of feces excreted, and in an apparently decreased percentage of lipids excreted when compared with the fecal excretion of the rats that received the basal starch diet.

SUMMARY

In two experiments in which rats were fed various adjuvants to basal diets, the feces were quantitatively collected and analyzed for fatty acids and non-saponifiable lipids.

In the first experiment, in which a basal sucrose diet that contained 10% soybean oil was fed, the addition of L-cystine, DL-methionine, ascorbic acid, bile salts, sodium fluoride, carotene, calcium carbonate, chloride or lactate had no significant effect upon the percentage of fecal excretion or the percentage retention of lipids.

The percentage excretion of feces was increased by the inclusion of 20% charred alfalfa or 10% agar-agar and by the substitution of lactose for the sucrose in the diet. The apparent percentage retention of total lipids was decreased by the inclusion of the charred alfalfa and increased by feeding 30% of soybean oil. The elimination of added soybean oil from the diet resulted in an apparent negative retention of lipids.

In the second experiment, in which starch replaced the sucrose of the basal diet, the addition of niacin or choline chloride had no effect on fecal elimination or retention of lipids. However, in this experiment the addition of carotene at three levels resulted in a significantly increased percentage of fecal elimination and in a significantly decreased percentage of total lipids apparently retained.

LITERATURE CITED

AUGUR, VIRGINIA, HILDA S. ROLLMAN AND HARRY J. DEUEL, JR. 1947 The effect of lecithin on the coefficient of digestibility and the rate of absorption of fat. J. Nutrition, *33:* 177.

BARNES, R. H., MARGARET F. PRIMROSE AND G. O. BURR 1944 The influence of the protein content of the diet upon fat digestibility. Ibid., *27:* 179.

BLOOR, W. R. 1938 Intermediary metabolism of the lipids. Oil and Soap, *15:* 63.

———— 1939 Fat transport in the animal body. Physiol. Rev., *19:* 557.

BOYD, OSCAR F., CARLOS F. CRIM AND J. F. LYMAN 1932 The absorption of calcium soaps and the relation of dietary fat to calcium utilization in the white rat. J. Biol. Chem., *95:* 29.

BURR, G. O., AND R. H. BARNES 1943a Fat metabolism. Annual Rev. Biochem., *12:* 157.

———— 1943b Noncaloric functions of dietary fats. Physiol. Rev., *23:* 256.

CROCKETT, MARY E., AND HARRY E. DEUEL, JR. 1947 A comparison of the coefficient of digestibility and the rate of absorption of several natural and artificial fats as influenced by the melting point. J. Nutrition, *33:* 187.

DEUEL, HARRY J., JR., AND LOIS F. HALLMAN 1940a The rate of absorption of synthetic triglycerides in the rat. Ibid., *20:* 227.

DEUEL, HARRY J., JR., LOIS F. HALLMAN AND ALVIN LEONARD 1940b The comparative rate of absorption of some natural fats. Ibid., *20:* 215.

ECKSTEIN, HENRY C. 1941 Lipid metabolism. Annual Rev. Biochem., *10:* 181.

FISCHER, JESSIE E., AND T. S. SUTTON 1949 Effects of lactose on gastrointestinal motility. A review. J. Dairy Sci., *32:* 139.

FRAZER, A. C. 1946 The effect of choline on the intestinal absorption of fat. Nature, *157:* 144.

HOAGLAND, R., AND G. G. SNIDER 1940 Nutritive properties of certain animal and vegetable fats. U. S. Dept. Agr. Tech. Bull. 725, p. 12.

———— 1943 Digestibility of some animal and vegetable fats. J. Nutrition, *25:* 295.

LEVIN, H., AND A. H. SMITH 1927 Growth on diets rich in fat. J. Biol. Chem., *72:* 222.

MATTIL, K. F. 1946 The digestibility of fats. A correlation of experimental data. Oil and Soap, *23:* 344.

MATTIL, K. F., AND J. W. HIGGINS 1945 The relation of glyceride structure to fat digestibility. J. Nutrition, *29:* 255.

SCHULZ, J. A., AND B. H. THOMAS 1945 The effect of various adjuvants to the diet of rats on the changes in body fats induced by feeding soybean oil. Iowa Agr. Exp. Sta. Res. Bull. 336, p. 628.

STEENBOCK, H., M. ERWIN, A. R. KEMMERER, V. M. TEMPLIN AND J. H. WEBER 1935 Fats play important role in animal nutrition. Wis. Agr. Exp. Sta. Bull. 430, p. 127.

WIKOFF, I. H., S. D. KOONCE AND H. J. MCGUIRE 1943 Some effects of high fat diets on intestinal elimination. Am. J. Digest. Dis., *10:* 266.

PEPTIDE WASTAGE CONSEQUENT TO THE INFUSION OF TWO PROTEIN HYDROLYSATES [1]

HALVOR N. CHRISTENSEN

Department of Biochemistry and Nutrition, Tufts College Medical School, and the New England Center Hospital, Boston, Massachusetts

(Received for publication May 15, 1950)

As adequate calorie nutrition by the intravenous route becomes feasible through the use of emulsified fat, the development of nutritionally perfect amino acid preparations becomes of greater importance. In an effort to aid progress in this direction, the disposition in man has been studied of the peptides of two protein hydrolysates in addition to those the author and his associates reported earlier (Christensen et al., '46, '47). The results emphasize again the extensive wastage of amino acids by way of the urine, which was previously demonstrated to result from the presence of peptides in the infusate. The importance of this wastage may be out of proportion to its size if critical amounts of an essential amino acid are lost. For example, hydrolysate "C," of unidentified origin (Silber and Porter, '49) and containing about 45% of its amino acids in peptide form, showed excellent biological value for dogs when fed by mouth but very little value when injected by vein. About 45% of the peptide nitrogen was lost into the urine as a result of the infusion.

The first of the two preparations studied in the present work was prepared by hydrolyzing fibrin by acid until only about 4% of the amino acids were in the bound form.[2] The

[1] Assisted by grants from the Abbott Laboratories, Inc., and the Baxter Laboratories, Inc.

[2] This hydrolysate was supplied by Dr. Douglas V. Frost of the Abbott Laboratories.

loss of peptides after infusion was found to represent about one-third of the small amount of peptide nitrogen injected. The other hydrolysate [3] was prepared by the action of pancreatic enzymes on bovine plasma. Of the amino acid nitrogen in this hydrolysate, 46.8% remained in bound form. Thirty-one to 32% of these bound amino acids were found to be lost in the urine as a result of the infusions.

EXPERIMENTAL

The subjects studied were either convalescent patients about to be discharged (removal of herniated nucleus pulposus, two; removal of pilonidal cyst, two; unexplained headache, one) or normal persons. All samples of the fibrin hydrolysate belonged to a single lot, dissolved in 5% glucose and containing, according to our analyses, 5.17 gm of free and only 0.20 gm of combined α-amino acid nitrogen per liter. The bovine plasma protein hydrolysate was also of a single lot, dissolved in water. In this preparation we found 3.23 gm of free and 2.84 gm of bound α-amino nitrogen per liter. The values for urinary free and bound α-amino nitrogen were corrected for the basal excretions shown during a 12-hour control period. The experimental urine samples represented the period of the infusions and the succeeding 4 hours. The analytical procedures and calculations have been described previously (Christensen et al., '46).

RESULTS AND DISCUSSION

The infusion rates, the increments of plasma amino acid and peptide concentration, and the increments in urinary excretion of free and combined amino acids are recorded in table 1. When the fibrin hydrolysate was infused, no increase in bound amino acids in the plasma was detected, although the increase of urinary conjugates was equivalent to about one-third of the small amount of peptide nitrogen injected. Associated with the increased hydrolysis of fibrin which reduced the content from 63.5–40% down to 4% of bound amino

[3] Hydrolysate supplied by Dr. Daniel Borgen of the Baxter Laboratories.

TABLE 1

Peptidemia and urinary wastage of amino acids as a result of the infusion of incomplete hydrolysates of protein

NO.	HYDROLYSATE	RATE OF INFUSION IN MG OF α-AMINO N PER KG PER HR.	TYPE OF α-AMINO N	ACCUMULATION IN PLASMA		AMOUNT INFUSED	EXTRA URINARY EXCRETION	WASTAGE
				End of infusion	Subsequently			
				mg %	*mg %*	*mg*	*mg*	*%*
1.	Fibrin	33	Free	5.0	0.08 (75 min.)	5,170	324	6.3
			peptide	0.0	...	200	60	30
2.	Fibrin	22	Free	3.46	0.22 (90 min.)	5,170	210	4.1
			peptide	0.0	0.0 (90 min.)	200	73	37
3.	Plasma proteins	53	Free	8.35	2.55 (60 min.)	3,230	257	7.9
			peptide	9.2	2.3 (60 min.)	2,840	902	32
4.	Plasma proteins	40	Free	6.1	0.78 (90 min.)	3,230	166	5.1
			peptide	7.5	1.6 (90 min.)	2,840	905	32
5.	Plasma proteins	28	Free	8.0	0.93 (230 min.)	3,230	185	5.7
			peptide	3.3	0.3 (230 min.)	2,840	876	31
6.	Plasma proteins	13	Free	1.5	...	1,630	70.5	4.3
			peptide	0.6	...	1,430	415	29

acids, there was a minimum urinary wastage of peptides. It is worth noting that the urinary loss of free amino acids was only about half as great as that observed earlier with the least-hydrolyzed fibrin employed in the present series of studies (Christensen et al., '47).

The peptide-rich hydrolysate of bovine plasma proteins gave concentrations of peptide nitrogen in the plasma as high as 9.2 mg per 100 ml, the concentration reached being dependent upon the infusion rate. More persistent elevations of plasma free amino acids (table 1) were obtained with this than with other preparations that have been studied. This behavior is undoubtedly related to the assimilation and utilization of amino acids by the cells (Christensen, Streicher and Elbinger, '48), but the nutritional consequence has not been defined. The urinary excretion of peptides was almost constant at 31 to 32% of the amount infused and strikingly independent of the degree of peptidemia, the infusion rate or the dose (cf. Christensen et al., '47; Silber and Porter, '49). When the infusion rate was quartered, the loss was still 29%. The peptides infused seem to fall sharply into two classes, one utilized and the other not, with few if any intermediate, slowly utilized peptides.

This wastage of 31% of the peptides of the latter hydrolysate is not as severe as the losses of about 45% which we observed with another hydrolysate (Christensen et al., '46). As a result of the larger content of peptides in the plasma protein preparation, however, the losses attributable to the presence of peptides in the two hydrolysates were about equal, being approximately 1 gm of α-amino nitrogen in every 6 or 7. Such losses are probably not of critical importance, unless essential amino acids are involved disproportionately. The desirability of overcoming technical obstacles to more complete hydrolysis appears, however, to be evident.

SUMMARY

A study of two protein hydrolysates — one of fibrin, nearly peptide-free, the other of bovine plasma proteins, very rich

in peptides — with respect to urinary losses upon intravenous infusion into man again indicates the superior retention of amino acids in the free state. The peptide-rich hydrolysate produced plasma peptide nitrogen values as high as 9.2 mg per 100 ml.

ACKNOWLEDGMENTS

The author is indebted to Dr. Stuart Welch and Dr. William Sweet for arrangements permitting studies of their convalescent patients at the New England Center Hospital, to these patients for their generous cooperation, and to Dr. Peter Mason and Miss Nancy E. Ray for technical assistance.

LITERATURE CITED

CHRISTENSEN, H. N., E. L. LYNCH, D. G. DECKER AND J. H. POWERS 1947 The conjugated, non-protein amino acids of plasma. IV. A difference in the utilization of the peptides of hydrolysates of fibrin and casein. J. Clin. Invest., *26:* 849.

CHRISTENSEN, H. N., E. L. LYNCH AND J. H. POWERS 1946 The conjugated, non-protein amino acids of plasma. III. Peptidemia and hyperpeptiduria as a result of the intravenous administration of partially hydrolyzed casein (Amigen). J. Biol. Chem., *166:* 649.

CHRISTENSEN, H. N., J. A. STREICHER AND R. L. ELBINGER 1948 Effects of feeding individual amino acids upon the distribution of other amino acids between cells and extracellular fluid. Ibid., *172:* 515.

SILBER, R. H., AND C. C. PORTER 1949 Urinary excretion of amino acids and peptides by dogs fed protein hydrolysates or amino acids. J. Nutrition, *38:* 155.

PANTOTHENIC ACID STUDIES

X. THE INFLUENCE OF A PANTOTHENIC ACID CONJUGATE (PAC) UPON GROWTH AND CITRATE FORMATION IN RATS [1]

TSOO E. KING,[2] F. M. STRONG AND VERNON H. CHELDELIN [3]
Department of Biochemistry, College of Agriculture, and Enzyme Institute, University of Wisconsin, Madison

THREE FIGURES

(Received for publication May 22, 1950)

A pantothenic acid conjugate described by the authors (King, Locher and Cheldelin, '48; King, Fels and Cheldelin, '49) has been found in the non-dialyzable fractions of tissues. It contains glutamic acid and adenine which, with free pantothenic acid, can be released by appropriate degradation procedures. Although the conjugate (PAC) is inactive for lactic acid bacteria, it can be rendered utilizable after liberation of the pantothenic acid moiety through the action of alkaline phosphatase and an unidentified liver enzyme. In this respect it is similar to the coenzyme A of Lipmann and co-workers (Novelli, Kaplan and Lipmann, '49), although several studies have shown the two conjugates to be different (King, Fels and Cheldelin, '49) and to coexist in animal tissues, particularly in heart muscle (Nishi, King and Cheldelin, '50).

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by grants from the Division of Research Grants and Fellowships, National Institutes of Health, Public Health Service, and the Nutrition Foundation, Inc. A part of the present paper was presented before the American Institute of Nutrition meeting at Atlantic City, New Jersey, April 1950 (Fed. Proc., *9:* 363).

[2] National Institute of Health Fellow, on leave 1949–50 from the Department of Chemistry, Oregon State College.

[3] Rockefeller Fellow, on leave 1949–50 from the Department of Chemistry, Oregon State College.

PAC possesses growth-promoting activity superior to that of the free vitamin for *Acetobacter suboxydans, Aerobacter aerogenes,* and *Escherichia coli,* and it is also a superior agent for overcoming the effect of an inhibitory analogue of the vitamin in *A. suboxydans* (King and Cheldelin, '50). Finally, when rats are placed on a pantothenic acid-free diet, PAC is conserved in the tissues at the expense of the free vitamin, to a degree equal to or greater than that observed for coenzyme A (Nishi, King and Cheldelin, 50).

The above observations point to the importance of PAC as a metabolically active form of pantothenic acid, despite the fact that this conjugate does not share with coenzyme A (Neilands, Higgins, King, Handschumacher and Strong, '50; Cheldelin, Teply and Green, '49) the property of acetylating sulfanilamide or choline. In an effort to document the properties of this conjugate further it was considered desirable to test its availability to pantothenic acid-deficient rats, when injected intraperitoneally or fed orally. The results, described below, indicate that PAC promotes growth fully as well as pantothenic acid when administered by either route, and that it is converted nearly as effectively as the free vitamin into coenzyme A-active compounds which catalyze citrate formation in the liver.

EXPERIMENTAL

Care of animals

Eighty albino rats of the Sprague-Dawley strain were used in this study. They were born on the same day and were maintained in litters with their mothers until they were 21 days old. They were divided for the experiment into 11 groups, each of which contained 6 to 10 animals, with equal numbers of males and females. The rats were housed in individual cages with wire-mesh bottoms. Body weights were recorded three times a week. Food and distilled water were allowed ad libitum.

The animals were first subjected to a preliminary 10-day depletion period, during which the basal diet (table 1) with-

TABLE 1

Composition of basal diet

Casein (vitamin free)	900 gm
Sucrose	3,400 gm
Mazola	450 gm
Sulfasuxidine	50 gm
Cod liver oil	50 gm
Salt mixture	200 gm
α-Tocopherol	1,000 mg
Menadione	50 mg
Choline chloride	5,000 mg
Inositol	2,000 mg
Thiamine chloride	40 mg
Riboflavin	80 mg
Nicotinic acid	500 mg
Pyridoxine hydrochloride	40 mg
p-Aminobenzoic acid	250 mg
Folic acid	10 mg
Biotin	0.2 mg

TABLE 2

Supplements to the basal diet for various groups of rats

GROUP	NO. OF RATS	SUPPLEMENTS ADMINISTERED		AMOUNT
		Route	Form	
				γ
A	10 (no. 201–210)	....	....	0
B	8 (no. 211–218)	intp.[1]	PA[2]	25
C	8 (no. 219–226)	intp.[1]	PA	50
D	8 (no. 227–234)	intp.[1]	PA	100
E	8 (no. 235–242)	intp.[1]	PAC[3] (no. 4–3)	700
F	8 (no. 243–250)	intp.[1]	PAC[3] (no. 4–3)	1,400
G	6 (no. 251–256)	oral[4]	PA	5
H	6 (no. 257–262)	oral[4]	PA	10
I	6 (no. 263–268)	oral[4]	PA	20
J	6 (no. 269–274)	oral[4]	PAC[5] (no. 28–4)	200
K	6 (no. 275–280)	oral[4]	PAC[5] (no. 28–4)	400

[1] Supplements injected intraperitoneally in the amounts indicated per rat per day.

[2] PA = pantothenic acid (calcium salt).

[3] PAC = pantothenic acid conjugate; preparation no. 4–3.

[4] Supplements administered orally in the amounts indicated per gram of diet.

[5] Preparation no. 28–4.

out B vitamins was given to all groups. During the next 9 days all of the B vitamins, with the exception of pantothenic acid, were given as is indicated in table 1. Then the respective supplements as shown in table 2 were supplied.

The menadione and the B vitamins listed in table 1 were supplied in a dry form which was prepared as follows: 1 ml of biotin solution containing 2 mg biotin was mixed with about 150 gm of sucrose and dried over P_2O_5 in a vacuum desiccator. The other vitamins were then mixed with the sucrose. The mixture was ground into a fine powder and "washed" out of the mortar by being ground three times with sufficient sucrose to provide a final weight of 300 gm. This mixture, which was sufficient for 50 kg of ration, was stored at $-5°C$.

Calcium pantothenate or PAC was incorporated into the diet in a dry mixture of sucrose containing 1 mg of the vitamin or 200 mg of the conjugate per gram. The amount of sucrose finally added to the diet was corrected for the amount added with the vitamins. The solutions for injection were prepared in water, adjusted to pH 7.0 with HCl or $NaHCO_3$ to contain a daily dose in 0.5 ml, and sterilized at 120°C. for 10 minutes.

PAC was prepared essentially as has been previously described (King, Fels and Cheldelin, '49). Preparation 28–4 contained 25 μg of bound pantothenic acid per milligram and 4–3 contained 50 μg per milligram. None of the activity was due to coenzyme A or free pantothenic acid, as judged by the inability of these samples to acetylate sulfanilamide or to promote growth in *Lactobacillus arabinosus*. The listed contents of bound pantothenic acid were obtained by microbiological assay with *Acetobacter suboxydans*, using as a standard a preparation (sample 801) which had been previously assayed with *L. arabinosus* 17–5 (Hoag, Sarett and Cheldelin, '45), after digestion with alkaline phosphodiesterase and an avian liver enzyme (Nielands and Strong, '48; Novelli, Kaplan and Lipmann, '49; Nishi, King and Cheldelin, '50). Pantothenic acid values so obtained are generally maximum compared to

those resulting from other digestion processes, and they have therefore been used as reference values in the present study, against which the rat performances are compared.

Citrate formation

At the end of the growth experiment some of the rats were sacrificed for the estimation of citrate formation. The animals were stunned by a blow on the head. The livers were removed immediately, packed in ice and weighed. They were masticated to a fine pulp in a Potter-Elvehjem homogenizer or in a Waring Blendor at 0–5°C., using a suitable amount of cold 0.9% KCl. The mixture was then made up to 200 mg of wet tissue per milliliter. An aliquot was taken for dry weight determination (95°C.).

All the preparations were tested within half an hour after the death of the animals. The amount of homogenate corresponding to 100 to 300 mg of wet tissue was incubated in a 50-ml Erlenmeyer flask with a concentration of 0.02 M sodium oxalacetate, 0.02 M $MgSO_4$, 0.015 M phosphate buffer (pH 7.5) and 0.01 M sodium acetate in a 5-ml total volume. The flasks were shaken at room temperature (27°C.) for 60 minutes.

Oxalacetic acid was prepared by Heidelberger's modification ('49) of Krampitz and Werkman's method ('41). The product (m.p. 149°, corrected) was stored in a desiccator in a cold room. It was dissolved and neutralized immediately before use.

Citrate was determined as previously described (Neilands, Higgins, King, Handschumacher and Strong, '50). At the end of incubation the enzymatic reaction was halted by the addition of 5 ml of 10% trichloroacetic acid.

RESULTS AND DISCUSSION

Two preparations of PAC containing 2.5 and 5.0% of bound pantothenic acid were tested at two levels orally and intraperitoneally. The rats which received doses of 0, 5, 10 and

20 μg of pantothenic acid per gram of diet were used as the standards for oral administration (cf. fig. 2). Similarly, those receiving 0, 25, 50 and 100 μg per day intraperitoneally (assuming a 5-gm daily food intake) were the standards for the injected animals (cf. fig. 1). The individual differences in body weight among the animals of the same sex in a given group were less than 10% of the means except during the last weeks, when the differences rose to about 15%.

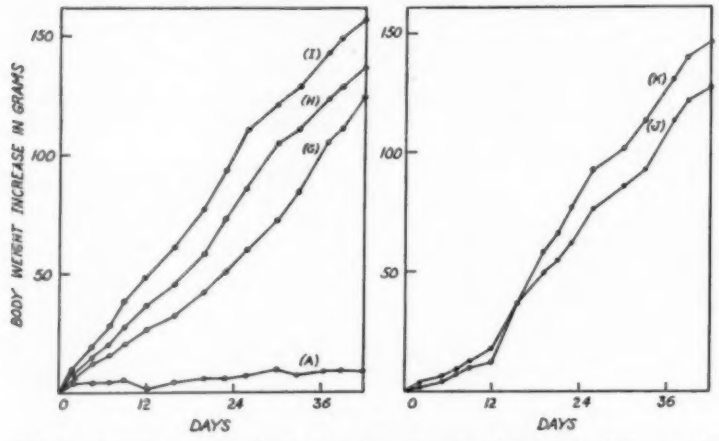


Fig. 1 Growth response of rats to intraperitoneally injected pantothenic acid and pantothenic acid conjugate (PAC). Curve numbers refer to group numbers cited in table 2.

The activity of PAC in terms of pantothenic acid was determined graphically. "Standard curves," one of which is shown in figure 3, were obtained at any time during the course of the experiment by plotting the mean growth of each group of standard animals as a function of the pantothenic acid administered. The mean weights of the PAC groups were then evaluated in terms of their equivalent pantothenic acid responses.

The body weight increases in the rats which received 100 μg of pantothenic acid by injection of a single daily dose were

inferior to those of the animals which received 20 μg orally per gram of food. Conversely, a 25-μg injected dose produced greater growth than the supposed oral equivalent of 5 μg per gram of food. Two explanations may be offered for this: first, it is possible that the high-vitamin rats ate more than 5 gm of food per day, and that the 100-μg dose of pantothenic acid was not optimum, whereas the food intake of the low-dietary vitamin group may have been less than 5 gm per day. It is

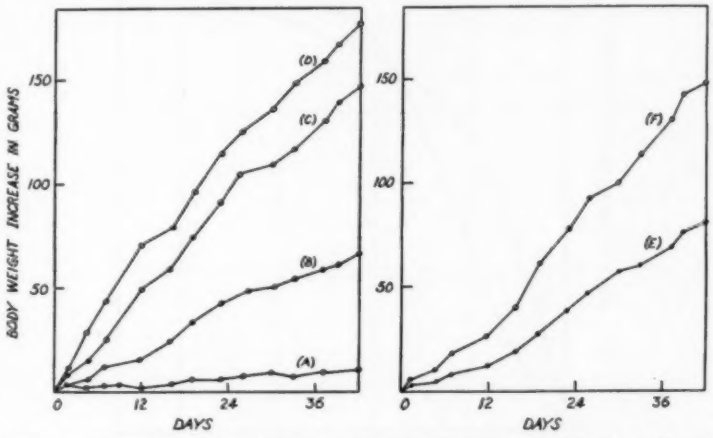


Fig. 2 Growth response of rats to orally administered pantothenic acid and PAC. Curve numbers refer to group numbers cited in table 2.

also possible that the total absorption of 100 μg of pantothenic acid after a single injection was poorer than that obtained by gradual intake through the alimentary tract. These differences appear to be of minor importance to the present study, however, since equivalent quantities of pantothenic acid and PAC were administered to the respective groups.

The relative effectiveness of PAC upon growth at various times during the course of the experiment is shown in table 3. During the early stages PAC was not as active as pantothenic acid, but it became fully as effective as the vitamin after three weeks. This change appears to be a real phe-

nomenon, for the slopes of the respective growth curves of animals in the pantothenic acid groups remained nearly constant throughout the experimental period, indicating that the pantothenic acid requirements were not changing materially. The reason for the gradual improvement is not known,

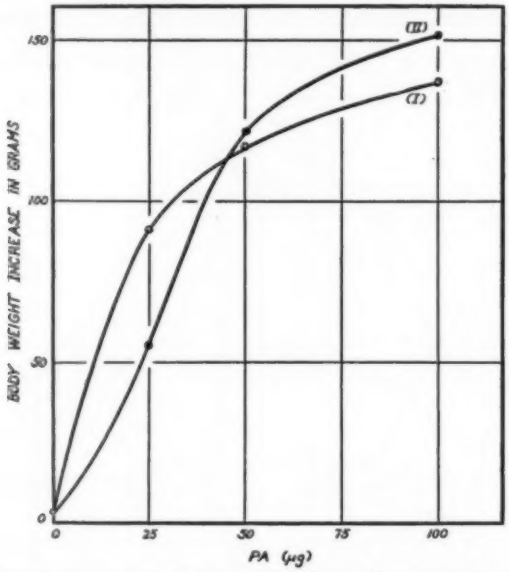


Fig. 3 Growth response of rats to graded doses of pantothenic acid. Growth period = 23 days. Curve I, daily injection; curve II, oral administration in amounts indicated per 5 gm of ration.

but it may have been due to increased permeability and absorption or, perhaps more likely, to the development of adequate amounts of suitable enzymes for the utilization of PAC. In this connection it may be recalled that during a short (10-day) period, Hegsted and Lipmann ('48) found coenzyme A to be only about 60% as effective as pantothenic acid in chicks, when fed orally.

The findings with respect to the ability of the liver homogenates of the deficient, control and PAC animals to form

TABLE 3

The pantothenic acid activity of pantothenic acid conjugate (PAC) by rat assay

PAC SAMPLE		PANTOTHENIC ACID ACTIVITY													
				Animal assay											
				Duration of experiment (days)											
No. of prep.	Dose	Microbiological assay	Mode of administration	12		19		26		35		42		Ave.[4]	
	mg	*μg*		*μg*	% [1]	*μg*	% [1]	*μg*	% [1]	*μg*	% [1]	*μg*	% [1]	*μg*	% [1]
28–4	1.0 [2]	25	oral	19	76	19	76	25	100	28	112	31	124	28	112
28–4	2.0 [2]	50	oral	37	74	41	82	45	90	50	100	52	104	49	98
		Average			75		79		95		106		114		105
4–3	0.7 [2]	35	intraperitoneal	18	51	34	97	38	109	36	103	36	103	37	106
4–3	1.4 [2]	70	intraperitoneal	12	17	42	60	58	83	58	83	78	110	65	93
		Average			33		79		96		93		107		100

[1] Per cent = % of activity of PAC compared to equivalent amounts of pantothenic acid.
[2] Amount indicated per 5 gm of ration.
[3] Daily dose.
[4] Average of last three values.

citrate from acetate and oxalacetate are summarized in table 4. It will be seen that the livers of the deficient animals (compared on a dry weight basis) were definitely inferior. Olson, Hirsch, Richards and Stare ('49) have observed similar results in the ventricle homogenates of ducklings. This was apparently due to the low coenzyme A content in the liver of the deficient animals. PAC-fed animals were as efficient

TABLE 4

Citrate formation by liver homogenates from rats of various groups

RAT NO.	SUPPLEMENT TO BASAL DIET	CITRIC ACID FORMED (MICROMOLS)	
		Per gm of wet liver per hour	Per 100 mg of dry tissue per hour
203	0 (PA-deficient)	24	
227	100 μg PA [1] injected, daily	45	
228	100 μg PA injected, daily	43	
246	1,400 μg PAC [2] injected, daily	42	
204	0 (PA-deficient)	28	
219	50 μg PA injected, daily	49	
229	100 μg PA injected, daily	52	
244	1,400 PAC [2] injected, daily	46	
201, 207	0 (PA-deficient)	31	6.1
220, 226	50 μg PA injected, daily	43	8.7
230, 234	100 μg PA injected, daily	51	10
243, 250	1,400 PAC [2] injected, daily	42	8.5

[1] PA = pantothenic acid; PAC = pantothenic acid conjugate.

[2] One thousand four hundred micrograms of PAC contain 70 μg of bound pantothenic acid.

as those supplemented with the free vitamin with respect to citrate formation, although the activity of PAC in the formation of citrate *in vitro* (in the presence of a dialyzed pigeon liver apoenzyme) was insignificant. The above findings suggest that in the pantothenic acid-deficient rat, PAC can give rise to a compound with coenzyme A activity. However, it is not known at present whether the conversion is direct or whether PAC is first broken down to pantothenic acid.

The degree of difference in citrate formation among the animals which received graded amounts of pantothenic acid

was not as great as differences in body weight. The rats receiving 50 μg of pantothenic acid daily showed about the same activity as those receiving 100 μg. This may be a reflection (in deficient animals) of superior conservation of the vitamin by the enzyme system which effects citrate formation.

The pantothenic acid contents of many tissues determined by earlier methods of microbiological assay were usually lower than those found by animal tests. The reason for such discrepancies has been made apparent with the discovery of the non-utilizability of coenzyme A and PAC by the common assay organisms, and digestion with alkaline phosphodiesterase and an avian liver enzyme has been found satisfactory for the release of the vitamin from these bound forms (Neilands and Strong, '48; Novelli, Kaplan and Lipmann, '49; Nishi, King and Cheldelin, '50). In spite of this improvement in assay procedure, an animal assay still appears desirable for reference purposes, for it has recently been found that a part of the pantothenic acid in liver is so firmly bound to the protein that it is not liberated by repeated extraction with trichloroacetic acid. Although this tightly bound form can be detected by prolonged hot water extraction, a question arises as to the nutritional availability of such material. This question can probably be answered best through animal assay. The growth curves of animals which received graded amounts of pantothenic acid, such as are shown in figure 1, should permit determination of the vitamin with fair accuracy.

SUMMARY

1. PAC administered either intraperitoneally or orally was as active as free pantothenic acid in supporting the growth of young albino rats.

2. The liver homogenates of rats grown on a pantothenic acid-deficient diet showed a reduced capacity for the formation of citrate. The livers of PAC-supplemented rats showed the same restoration of this activity as those of animals supplemented with the free vitamin.

3. The degree of difference in the ability to form citrate among the animals receiving graded amounts of pantothenic acid was not as great as the differences in body weight.

ACKNOWLEDGMENTS

The authors wish to acknowledge the gift of the B vitamins used in this study from Merck and Company, Inc.; the assistance in caring for the animals of C. J. Wattenberger and J. A. Berard; and helpful discussions with Dr. C. A. Baumann.

LITERATURE CITED

CHELDELIN, V. H., L. TEPLY AND D. E. GREEN 1949 Unpublished observation.

HEIDELBERGER, C. 1949 Private communication.

HEGSTED, D. M., AND F. LIPMANN 1948 The pantothenic acid content of coenzyme A by chick assay. J. Biol. Chem., *174:* 89.

HOAG, E. H., H. P. SARETT AND V. H. CHELDELIN 1945 Use of *Lactobacillus arabinosus* 17–5 for microassay of pantothenic acid. Ind. Eng. Chem., Anal. Ed., *17:* 60.

KING, T. E., AND V. H. CHELDELIN 1950 Pantothenic acid studies. VIII. Growth of microorganisms and counteraction of antimetabolites with a pantothenic acid conjugate (PAC). J. Bact., *59:* 229.

KING, T. E., I. G. FELS AND V. H. CHELDELIN 1949 Pantothenic acid studies. VI. A biologically active conjugate of pantothenic acid. J. Am. Chem. Soc., *70:* 131.

KING, T. E., L. M. LOCHER AND V. H. CHELDELIN 1948 Pantothenic acid studies. III. A pantothenic acid conjugate active for *Acetobacter suboxydans*. Arch. Biochem., *17:* 483.

KRAMPITZ, L. O., AND C. H. WERKMAN 1941 The enzymic decarboxylation of oxalacetate. Biochem. J., *35:* 595.

NEILANDS, J. B., H. HIGGINS, JR., T. E. KING, R. E. HANDSCHUMACHER AND F. M. STRONG 1950 The concentration of bound pantothenic acid. J. Biol. Chem., *185:* 335.

NEILANDS, J. B., AND F. M. STRONG 1948 The enzymatic liberation of pantothenic acid. Arch. Biochem., *19:* 287.

NISHI, H., T. E. KING AND V. H. CHELDELIN 1950 Pantothenic acid studies. IX. The influence of dietary pantothenic acid upon a pantothenic acid conjugate (PAC) in rat tissues. J. Nutrition, *41:* 279.

NOVELLI, G. D., N. O. KAPLAN AND F. LIPMANN 1949 The liberation of pantothenic acid from coenzyme A. J. Biol. Chem., *177:* 97.

OLSON, R. E., E. G. HIRSCH, H. RICHARDS AND F. J. STARE 1949 Coenzyme A and citrate formation in homogenates of heart ventricle from normal and pantothenic acid-deficient ducklings. Arch. Biochem., *22:* 480.

NUTRITIVE VALUE OF CANNED FOODS

XLII. A STUDY OF THE PROTEIN VALUE OF YOUNG AND MATURE PEAS (PISUM SATIVUM)[1]

R. G. CHITRE,[2] J. N. WILLIAMS, JR. AND C. A. ELVEHJEM
Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison

THREE FIGURES

(Received for publication May 29, 1950)

Investigations of the biological value of the protein in peas are comparatively few. Finks, Jones and Johns ('22) reported that peas *(Pisum sativum)* gave normal growth when included at a level of 75% in a ration for rats. They believed that peas supplied all the amino acids essential for growth. Cook and Robertson ('41) reported the Alaska peas have about 50% of the supplementary value of casein when added to a cereal ration for chicks. Woods, Beeson and Bolin ('43), investigating the protein value of peas *(Pisum sativum)*, concluded that methionine was the limiting factor for growth in rats. They also observed that baking or autoclaving decreased the protein value. Mitchell and Block ('46) believed that on the whole the proteins of peas possessed low biological value, which was further diminished by heat. Murray ('48) observed that canning has a very harmful effect on the pea

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported by a grant from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation, and by funds from the National Canners' Association, Can Manufacturers' Institute Nutrition Program, Washington, D. C.

[2] UNICEF fellow in nutrition, under the auspices of the United Nations World Health Organization. Present address: Seth. G. S. Medical College, Bombay, India.

protein, probably because the cystine content is lowered by this process. Another paper of interest in this connection is that by Richardson ('48).

The present investigation [3] was undertaken to study more completely the biological value of the protein in sweet peas *(Pisum sativum)*. Since various workers have obtained conflicting results concerning the effect of heat on the growth-promoting value of protein, the effect of heat-processing during canning was studied. In order to relate the essential amino acid content of peas to the biological value of the protein, the samples of peas were analyzed for their essential amino acid content. Knowledge of the amino acid composition thus obtained would also greatly facilitate the growth studies. Since changes in the protein content of plants have been observed to occur with development (Albaum and Cohen, '43), a comparison was made of the biological value of the protein in immature peas with that in nearly mature peas.

EXPERIMENTAL AND RESULTS

Preparation of samples

Samples of immature [4] and nearly mature sweet peas [5] were obtained immediately after the vined peas were size-graded at the cannery.[6] The immature peas were of the Loyalty variety and consisted of sieve size 4 and smaller ones; the nearly mature peas were of the Superior variety and consisted of sieve size 6. Samples of peas of both varieties were collected under the supervision of a representative of the nutritional program [7] and one of the authors, at the cannery. Samples of the raw peas were brought to drying ovens within

[3] These studies were undertaken as a part of the nutritional program of the National Canners' Association, Can Manufacturers' Institute.

[4] Tenderometer reading of these peas was 110.

[5] Tenderometer reading of these peas was 140.

[6] Both immature and nearly mature peas were obtained from Libby, McNeil and Libby at Lake Mills, Wisconsin.

[7] The authors are indebted to Dr. J. F. Feaster, supervisor of the biochemical group of the American Can Company, Maywood, Illinois, for his aid and cooperation in collecting the samples of peas.

an hour after the vined peas were delivered to the cannery, and dried to constant weight in a current of air at 50°C. (122°F.). Other samples from the same lots of peas were blanched at the cannery for two and one-half minutes at 85°C. (184°F.) and then canned and heat-processed in brine for 35 minutes at 116°C. (240°F.). The canned peas were drained of excess liquid through cheese cloth and dried to constant weight in air at 50°C. Each sample of both dried raw and canned peas was ground to a fine powder in a Wiley mill and stored in a waterproof carton until used.

Nitrogen and amino acid content of samples

To estimate the level of protein in the peas, total nitrogen was determined in each sample by a semi-micro Kjeldahl procedure. The protein level was calculated by multiplying total nitrogen by 6.25.

The essential amino acid content of the peas (including arginine) was determined by microbiological assay after acid or alkaline hydrolysis. For the acid hydrolysis, samples of the ground dried peas were autoclaved for 10 hours at 12 lb. pressure in 40 volumes of 3N HCl, and for alkaline hydrolysis samples were autoclaved for 15 hours at 12 lb. pressure in 20 volumes of 3N KOH. All of the amino acids determined except tryptophan were assayed in the acid hydrolysates. Because of the destruction of tryptophan during acid hydrolysis, it was assayed in the alkaline hydrolysate. Leucine, phenylalanine, tryptophan, and valine were determined with *L. arabinosus;* histidine, lysine, and isoleucine with *Leuconostoc mesenteroides* P-60; arginine with *L. delbruckii*-3; and threonine and methionine with *S. faecalis.* In all cases the medium of Henderson and Snell ('48) was employed.

In table 1 are shown the results of the protein and amino acid analyses. With both raw and canned peas the total protein ($N \times 6.25$) was found to be higher in the young peas than in the mature peas. In essential amino acid content, however, the immature peas were considerably below the

nearly mature peas, except for tryptophan, valine, arginine, and threonine. In the immature raw peas threonine assayed considerably higher than in the mature raw peas. In most cases the raw peas contained slightly more of the essential amino acids than the canned peas on a dry weight basis. It may also be observed from the table that the total protein of both immature and nearly mature canned peas was somewhat lower than that of both the samples of raw peas. The brine could not be adequately assayed for amino acids because of the toxicity of the salt content to microorganisms.

TABLE 1

The essential amino acid content of sweet peas (gm % on a dry weight basis)

AMINO ACID	RAW		CANNED	
	Nearly mature peas	Immature peas	Nearly mature peas	Immature peas
	Protein = 27.5%	Protein = 29.6%	Protein = 21.05%	Protein = 23.6%
Leucine	1.98	1.38	1.84	1.43
Phenylalanine	0.82	0.74	0.81	0.57
Tryptophan	0.20	0.20	0.20	0.22
Valine	0.91	0.91	0.80	0.80
Histidine	0.33	0.26	0.30	0.27
Lysine	0.88	0.42	0.65	0.51
Isoleucine	1.30	1.08	1.00	1.03
Arginine	2.00	2.00	1.84	1.83
Threonine	0.90	1.40	0.70	0.70
Methionine	0.22	0.18	0.20	0.23

Growth studies with albino rats

a. Growth rates on the different samples of peas. In order to test the growth-producing properties of the peas, they were fed to young albino rats at a level supplying 15% protein in a synthetic ration. The rations contained 2% of a vitamin mixture, as previously described by Williams and Elvehjem ('49), 4% salts IV as described by Phillips and Hart ('35), 5% corn oil, peas at the appropriate level, and sucrose to make 100%. The peas formed the sole source of protein in each ration, which amounted to 54% in the case

of nearly mature raw peas, 50.7% in the case of immature raw peas, 69.8% in the case of nearly mature canned peas, and 63.6% in the case of immature canned peas.

The level of peas incorporated into the rations was arbitrarily taken to supply 15% protein. Since all samples were

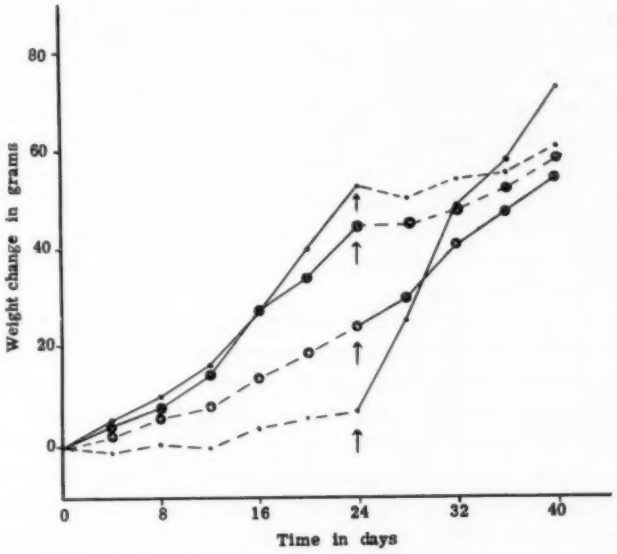


Fig. 1 Growth curves of rats, showing the relative biological value of nearly mature and immature raw and canned sweet peas.

—●— = nearly mature raw peas
—⊙— = nearly mature canned peas
– –●– – = immature raw peas
– –⊙– – = immature canned peas

fed at this level, differences in the biological values of the various samples of peas should be demonstrated, even though many of the essential amino acids would thus be fed at suboptimum levels (see table 1).

Groups of 6 weanling male albino rats of the Holtzman strain weighing 40 to 45 gm were placed in individual cages and given the respective ration and water ad libitum for

24 days. The animals were weighed on alternate days and a record of daily food intake was kept. After the 24-day feeding period the rats were interchanged on the various diets, as shown in figure 1, in order to decrease the effects of individual variation within the groups.

From the figure it may be seen that during the 24-day preliminary feeding period the immature canned peas supported much better growth than immature raw peas fed at the same nitrogen level. Because of the overlapping of the growth curves at 6 and 14 days for the rats fed the raw and canned nearly mature peas, any difference in biological value between the raw and canned nearly mature peas is doubtful. After interchanging the diets, a parallelogram-like effect was obtained in which the lower growth rate given by the immature peas was increased by feeding the nearly mature peas and vice versa.

When compared to the growth of weanling rats fed a good casein ration, i.e., 35 gm per week (Womack and Rose, '46), the best growth obtained in figure 1 was less than half the optimum growth with casein. This can probably be explained by the suboptimum levels of some of the essential amino acids in the rations (see table 1). However, since the pea rations were fed at isonitrogenous levels, differences in the biological values of the various samples of peas should be evident whether the amino acid levels were optimum or not.

The differences in growth on the different rations cannot be explained by differences in the amounts of food ingested, since in all cases the rats consumed practically the same amount of food each day regardless of the ration being fed

b. The effect of amino acid supplements. A second series of growth studies was carried out in which, first, the rations were supplemented with crystalline amino acids so that the amino acid contents of the two raw varieties and the two canned varieties of peas were made equal except for threonine. Each group of rats consisted of 6 animals, which were fed, watered, and weighed as before. In figure 2 it may be seen that supplementation of the immature raw pea ration with essential

amino acids did not remedy the low growth rate, although it was increased slightly over that reached by the rats on the unsupplemented ration (fig. 1).

At the end of the 16-day feeding period, the ration of the group fed the nearly mature raw peas was supplemented

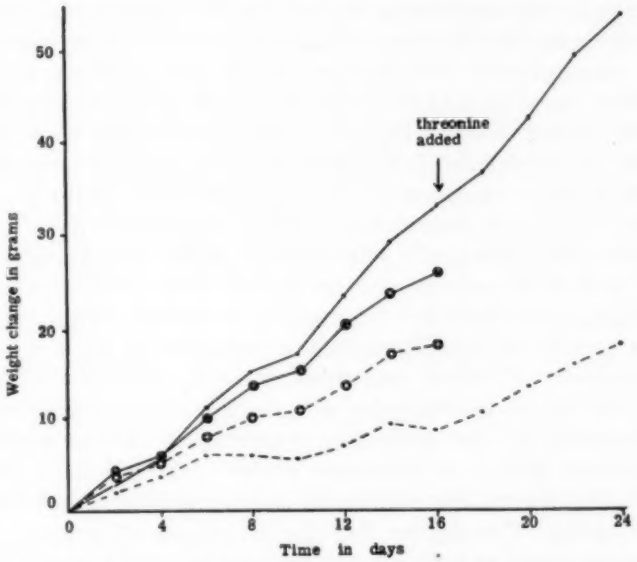


Fig. 2 Growth curves, showing the effect of supplementing young peas with amino acids to bring them to the level of mature peas in this respect. The effect of adding excess threonine to mature raw peas.

—●— = nearly mature raw peas
— —●— — = immature raw peas
—⊙— = nearly mature canned peas
— —⊙— — = immature canned peas

with an amount of threonine to make the intake of this amino acid the same as that of the group fed the immature raw peas. The results of these growth studies are included in figure 2; the arrow indicates when they were begun. Lack of effect of the threonine supplement indicated that a possible

threonine imbalance was not the cause of the poor response to the immature raw peas.

c. Effect of autoclaving or incubating the peas. It appeared from the foregoing results that other factors besides low amino acid levels or threonine imbalance were responsible for the poor growth-promoting properties of the immature raw peas as compared to the other peas. During the feeding experiments it had been observed that the rats receiving the immature raw peas carried considerable moisture to their rations, more so than the other groups of animals. Therefore it was possible that when the immature raw peas were moistened, an enzymatic mechanism was set up which decreased the protein value of the peas. Partial hydrolysis of the protein with subsequent deamination of the free amino acids could occur. The keto acids thus formed might be available for the growth of microorganisms in the amino acid assays, although unavailable for the growth of the rat. This hypothesis was tested first by incubating samples of the peas with an amount of water equivalent to that originally removed by drying. If considerable deamination of amino acids occurred during the incubation, a noticeable rise in ammonia and keto acid concentrations should take place. Pyruvic acid was chosen for estimation as an example of one of the keto acids. The figures for the formation of pyruvic acid were assumed to estimate the amount of deamination af alanine. The production of ammonia during incubation was used as an indication of the amount of general amino acid deamination. It is acknowledged that both pyruvic acid and ammonia could arise by routes other than those mentioned; however, the extent of their formation should give an indication of the general enzyme activity in the peas.

Samples of the peas employed in this study were diluted with an amount of water equal to the volume lost during drying. A portion of each sample was covered with toluene and incubated for 120 hours at 37°C. (99°F.). Another portion of each sample was autoclaved immediately at 15 lb. pressure for 30 minutes. The autoclaving was expected to

inactivate the enzymes in the peas and also to extract the water-soluble elements more completely than if the peas were simply extracted with cold water. Extracts of each of the incubated or autoclaved samples were then assayed for pyruvic acid (Elgart and Nelson, '41) and free ammonia (Folin and Macallum, '12).

In table 2 it may be observed that incubation of the peas with water produced a very large increase of both ammonia

TABLE 2

Pyruvic acid and ammonia content of the water extract from peas (incubated or autoclaved)

TYPE OF PEAS	SUBSTANCE MEASURED	AUTOCLAVED (15 LB. FOR 30 MIN.)	INCUBATED (120 HOURS)
Nearly mature (raw)	pyruvic acid (mg %)	162.8	212.5
Immature (raw)		282.6	503.5
Nearly mature (canned)		162.6	165.6
Immature (canned)		121.3	165.6
Nearly mature (raw)	ammonia (mg %)	177.0	241.0
Immature (raw)		824.0	2,054.0
Nearly mature (canned)		178.0	190.0
Immature (canned)		178.0	190.0

and pyruvic acid in the raw peas, while the canned peas produced relatively little of either product during incubation. Also far greater pyruvate and ammonia production occurred in the immature raw peas than in the nearly mature raw peas. On the basis of these results it appears that the enzymes producing pyruvate and ammonia were active in both the immature and nearly mature raw peas, but much more active in the former than in the latter. Moreover, even without incubation the immature raw peas possessed larger quantities of pyruvate and ammonia than the nearly mature

peas. In the canned peas a slight increase in the amounts of pyruvate and ammonia occurred during incubation. These increases, however, were probably due to greater extraction during the 120-hour incubation period than was possible in the case of the 30-minute autoclaving.

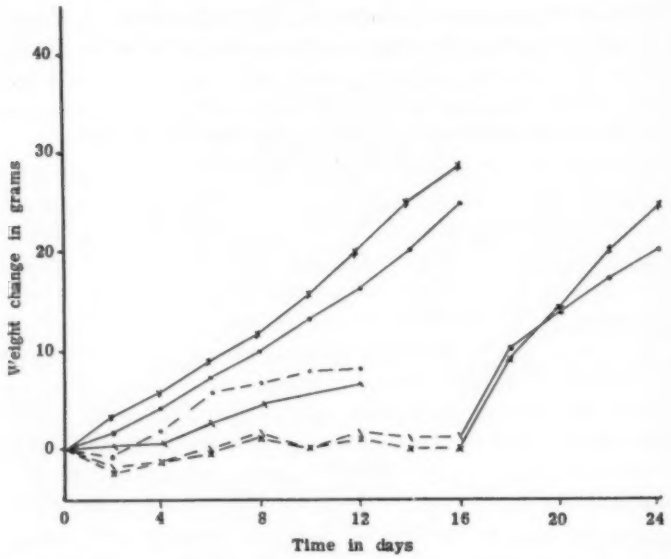


Fig. 3 Growth curves, showing the effects of incubating or autoclaving upon the biological value of peas.

—●— = nearly mature raw peas
– –●– – = immature raw peas
—×— = nearly mature raw peas (autoclaved)
– –×– – = immature raw peas (autoclaved)
—\— = nearly mature raw peas (incubated)
– –\– – = immature raw peas (incubated)

In order to test the effects of enzymatic action on the biological value of the peas, autoclaved and incubated samples of the immature and nearly mature raw peas were prepared as described above. The water or toluene added for the incubation or autoclaving was removed by drying the samples

at 50°C. in air. The samples were then mixed into rations and fed to the rats, as described earlier in this report. Control groups were also set up, and were fed the respective untreated samples of peas.

The results of these experiments, presented in figure 3, indicate that incubation of both the immature and nearly mature raw peas resulted in a depression of growth response in rats, indicating that enzymatic action was very important in decreasing the biological value of the protein in the two types of peas. Although it has been suggested that the enzymatic deamination of essential amino acids may have been responsible for these results, the possibility of an enzymatic liberation of a toxic principle, as in the case of soya beans, should not be overlooked. However, the fact that both ammonia and pyruvic acid were liberated in such significant amounts during incubation of the raw peas suggests that the deamination hypothesis is correct at least in part.

In the case of the autoclaved samples, somewhat unexpected results were obtained. The autoclaved nearly mature raw peas gave better growth than the unautoclaved samples, whereas autoclaving the immature raw peas decreased the growth value to the same level as that given by the incubated immature raw peas. No conclusive explanation can be given for these results, although at first hand it appears that autoclaving injures the protein, as far as biological value is concerned, in immature peas but not in nearly mature peas. This problem may be related to the digestibility of the pea protein for the rat, and consequently to amino acid availability. In both immature and nearly mature peas the availability of amino acids was possibly decreased, but the excess of dietary protein may have masked the effect in the nearly mature peas, while the decrease in biological value of the immature pea protein was enough to become limiting for growth in the rat.

In view of the recently reported work by Friedman and Kline ('50), it is possible that the glucose-amino acid complexes formed even at the relatively low temperatures used

for drying the present samples of peas decreased the availability of the essential amino acids to such an extent that the biological value was decreased, particularly in the immature raw peas. The fact that enzymes were still demonstrably active in the raw peas, especially in the immature raw peas, indicates that hydrolysis of the protein may have occurred, with subsequent reaction between the free amino acids and glucose. This would greatly decrease the availability of the amino acids to the rat, although, as Friedman and Kline demonstrated, not to the microorganisms used in the amino acid assays.

SUMMARY

The effects of canning on the biological value for young growing rats of the protein in peas have been investigated. Comparison of the biological value of protein in immature and nearly mature peas has also been made.

It appears that canning does not decrease the biological value of nearly mature peas, while it enhances that of immature peas. The results of other workers with respect to the detrimental effects of canning on the biological value of pea protein, cited in the introduction to this paper, could not be confirmed.

Investigations concerning the cause of the poor biological value of immature raw peas suggest that hydrolysis of the protein and subsequent deamination of its amino acids occur through enzymatic action when the peas are moistened. Canning destroys the enzyme activity. The possibility is discussed that reactions during the drying process between the sugar in the raw peas and the amino acids freed by proteolysis contribute to the decrease in the biological value of the raw peas for the rat.

A complete analysis of immature and nearly mature raw peas and immature and nearly mature canned peas for essential amino acid content has been presented.

LITERATURE CITED

ALBAUM, H. G., AND P. P. COHEN 1943 Transamination and protein synthesis in germinating oat seedlings. J. Biol. Chem., *149:* 19.

COOK, J. W., AND G. I. ROBERTSON 1941 Nutritive value of Alaska pea protein + DL-valine. Poultry Sci., *20:* 201.

ELGART, S., AND N. NELSON 1941 Elimination of acetoacetic acid in the determination of pyruvic acid by Lu's method. J. Biol. Chem., *138:* 443.

FINKS, A. J., D. B. JONES AND C. O. JOHNS 1922 The role of cystine in the dietary properties of the proteins of cow peas *(Vigna sinensis)* and of field peas *(Pisum sativum)*. Ibid., *52:* 403.

FOLIN, O., AND A. B. MACALLUM 1912 On the determination of ammonia in urines. Ibid., *11:* 523.

FRIEDMAN, L., AND O. L. KLINE 1950 The relation of the amino acid-sugar reaction to the nutritive value of protein hydrolysates. J. Nutrition, *40:* 295.

HENDERSON, L. M., AND E. E. SNELL 1948 A uniform medium for determination of amino acids with various microorganisms. J. Biol. Chem., *172:* 15.

MITCHELL, H. H., AND R. J. BLOCK 1946 Some relationships between the amino acid contents of proteins and their nutritive values for the rat. Ibid., *163:* 599.

MURRAY, HAZEL C. 1948 The biological value of the protein of field pea products, with a comparison of several methods used for this determination. J. Nutrition, *35:* 257.

PHILLIPS, P. H., AND E. B. HART 1935 The effect of organic dietary constituents upon chronic fluorine toxicosis in the rat. J. Biol. Chem., *109:* 657.

RICHARDSON, L. R. 1948 Southern peas and other legume seeds as a source of protein for the growth of rats. J. Nutrition, *36:* 451.

WILLIAMS, J. N., JR., AND C. A. ELVEHJEM 1949 The relation of amino acid availability in dietary protein to liver enzyme activity. J. Biol. Chem., *181:* 599.

WOMACK, M., AND C. W. ROSE 1946 Evidence for the existence of an unidentified growth stimulant in proteins. Ibid., *162:* 735.

WOODS, E., W. M. BEESON AND D. W. BOLIN 1943 Field peas as a source of protein for growth. J. Nutrition, *26:* 327.

THE PROTECTIVE ACTION OF VITAMIN B_{12} AGAINST THE TOXICITY OF DL-THYROXINE [1]

BARNETT SURE AND LESLIE EASTERLING
Department of Agricultural Chemistry, University of Arkansas, Fayetteville

(Received for publication May 27, 1950)

When rations containing soybean meal are employed, the retardation in growth caused by dessicated thyroid or iodinated casein has been counteracted in the rat and chick by crystalline vitamin B_{12} (Betheil and Lardy, '48; Emerson, '49; Nichol and associates, '47). In the present communication experimental evidence is presented on the basis of which it is evident that 0.2 to 0.5 μg daily of crystalline vitamin B_{12} not only protects against the entire collapse produced by subcutaneous injections of 0.2 mg daily of DL-thyroxine, but also permits considerable growth to take place.

Pregnant female albino rats were transferred from our stock colony ration several days before parturition to the following purified ration A (parts by weight), which allowed a minimum of storage of vitamin B_{12}: casein,[2] 30; cellu flour, 2; methionine, 0.3; Sure's salts no. 1 (Sure, '41), 4; dry brewers' yeast,[3] 1; wheat germ oil, 3; cod liver oil, 2; and cerelose, 57.7; all in grams. The following components of the vitamin B complex were given daily separately from the ration: thiamine, 50; riboflavin, 50; pyridoxine, 50; nicotinic acid, 50; calcium pantothenate, 300; biotin, 10; folic acid, 10, all in micro-

[1] Research paper no. 931, Journal Series, University of Arkansas. Published with the approval of the Director of the Arkansas Experiment Station. Supported by the Williams-Waterman Fund of the Research Corporation.

[2] Smaco. Extracted with 7 lots of hot ethyl alcohol, 8 hours to each lot.

[3] Anheuser-Busch, type G.

grams; and inositol, 2; *p*-aminobenzoic acid, 6; and choline, 9, in milligrams. On the day of parturition the concentration of these vitamins was increased as follows: thiamine, 100; riboflavin, 100, pyridoxine, 100; nicotinic acid, 100; calcium pantothenate, 600; biotin, 10; and folic acid, 80, in micrograms; and inositol, 3; *p*-aminobenzoic acid, 12; and choline chloride, 12, in milligrams. In addition, the females received three times weekly 6 drops of halibut liver oil and two drops of a tocopherol concentrate as supplements of vitamins A, D, and E. Successful rearing of young was obtained on this maternal diet. Weanling rats weighing about 45 gm each were transferred to two types of experimental diets: first, to the ration of Register, Ruegamer and Elvehjem ('49), designated in this paper as ration B, of the following composition: in grams, whole ground yellow corn, 46.35; commercial soybean meal, 46.35; corn oil,[4] 5; cystine, 0.3; $CaHPO_4$, 0.92; $CaCO_3$, 0.6; iodized sodium chloride, 0.44; $MnSO_4 \cdot 4H_2O$, 0.04; in milligrams thiamine, 0.3; riboflavin, 0.3; niacin, 2; pyridoxine, 0.2; pantothenic acid, 2; folic acid, 0.025; biotin, 0.01, inositol, 10; choline, 100; and *p*-aminobenzoic acid, 25. No iodinated casein was used in this ration, since the source of the thyrogenic factor was crystalline DL-thyroxine administered separately. Vitamins A and D were administered as oleum percomorphum diluted 1:4 with corn oil and fed at the rate of two drops per animal per week.

Another group of weanling rats, weighing about 46 gm each, were transferred to our purified ration C, of the following composition: casein,[5] hot alcohol-extracted, 18; cystine, 0.2; sulfasuxidine, 0.3; cellu flour, 2; Sure's salts no. 1, 4; corn oil,[6] 4; cod liver oil, 1, in grams; and cerelose, 70.5; thiamine, 3; riboflavin, 3; nicotinic acid, 3; calcium pantothenate, 5; *p*-aminobenzoic acid, 5; pyridoxine, 5; inositol, 20; and choline chloride, 100, in milligrams. Ten micrograms of folic acid and 10 μg of biotin were given daily to each animal separate from the ration.

[4] Mazola.

[5] See footnote 2, page 221.

[6] See footnote 4.

TABLE 1

Protective action of vitamin B_{12} against the toxicity of DL-*thyroxine* [1]

ANIMAL	DAYS ON EXPERIMENT	DAILY DOSE OF CRYSTALLINE VITAMIN B_{12}	BODY WEIGHT		
			Initial	Final	Change
		μg	*gm*	*gm*	*gm*
Males			*Soybean meal-corn ration B*		
1	8 — D [2]	0	63	64	+ 1
2	13 — D	0	53	68	+ 15
3	2 — D	0	44	41	— 3
4	2 — D	0	48	51	+ 3
5	5 — D	0	44	41	— 3
6	2 — D	0	48	51	+ 3
7	28	0.3	43	139	+ 96
8	28	0.3	43	151	+ 108
9	28	0.3	40	141	+ 101
10	28	0.3	42	139	+ 97
11	28	0.3	40	133	+ 93
12	28	0.3	42	151	+ 109
Females					
13	5 — D	0	48	38	— 10
14	2 — D	0	46	46	± 0
15	2 — D	0	42	47	+ 5
16	28	0.3	42	144	+ 102
17	28	0.3	41	121	+ 80
18	28	0.3	38	115	+ 77
Males			*Purified ration C*		
19	15 — D	0	50	64	+ 14
20	21 — D	0	50	65	+ 15
21	14 — D	0	46	55	+ 9
22	9 — D	0	46	46	± 0
23	22	0.3 to 0.5	44	105	+ 61
24	22	0.3 to 0.5	44	88	+ 44
25	22	0.3 to 0.5	44	90	+ 46
26	22	0.3 to 0.5	44	103	+ 59
Females					
27	15 — D	0	48	65	+ 17
28	11 — D	0	48	54	+ 6
29	14 — D	0	45	53	+ 8
30	13 — D	0	45	52	+ 7
31	19	0.3 to 0.5	46	92	+ 46
32	19	0.3 to 0.5	46	92	+ 46
31	19	0.3 to 0.5	46	93	+ 47
32	19	0.3 to 0.5	46	92	+ 46

[1] Thyroxine (0.2 mg) was injected subcutaneously daily into each animal.
[2] Died.

Litter mates of the same sex were fed the above rations ad libitum and each animal was injected subcutaneously daily with 0.2 mg of crystalline DL-thyroxine. One animal was the negative control and received no vitamin B_{12} and a litter mate served as the positive control, which received a protective dose of 0.3 to 0.5 μg daily of crystalline vitamin B_{12}. The results are summarized in table 1.

It will be noted that weanling rats transferred from maternal purified ration A to the soybean-corn ration B and injected daily with 0.2 mg of DL-thyroxine had a very short survival period. Out of 9 animals, 5 died in two days, two succumbed in 5 days, one in 8 days, and one in 13 days. The latter two animals weighed appreciably more at the beginning of the experiment and probably had more storage of vitamin B_{12}. The litter mates which received the same daily dose of thyroxine had a supplementary daily administration of 0.3 μg of vitamin B_{12}, which permitted not only 100% protection against the toxicity of the thyroxine but also allowed considerable growth. During a period of 28 days the animals which received the minute amounts of vitamin B_{12} trebled their body weights. The ratio of vitamin to hormone was 0.3 μg to 200 μg; in other words, it took one part of vitamin B_{12} to protect against the toxicity of 666 parts of the thyroid hormone, thyroxine.

On the purified casein ration, with the same daily dose of 0.2 mg thyroxine, the average survival was 14 days. While 0.3 μg daily of vitamin B_{12} allowed 100% protection against the poisonous effect of thyroxine, it was necessary on the 7th day of the experiment to increase the daily dose of vitamin B_{12} to 0.5 μg to permit growth to proceed. During a period of 19 days the animals which received the minute doses of vitamin B_{12} doubled or more than doubled their body weights.

Undoubtedly, in hyperthyroidism there are greater requirements for several components of the vitamin B complex, among them vitamin B_{12}. That as little as one part of this vitamin can produce 100% protection against 666 parts

of the thyroid hormone, thyroxine, is noteworthy, and such results may prove of clinical interest in connection with treatment of the toxic goiter associated with a high basal metabolic rate.

SUMMARY

As little as one part of crystalline vitamin B_{12} affords 100% protection against a toxic fatal dose of 666 parts of the thyroid hormone, thyroxine; i.e., 0.3 μg vitamin B_{12} against 200 μg thyroxine.

ACKNOWLEDGMENTS

We wish to express our appreciation for the generous gifts of crystalline vitamin B_{12} from Merck and Co., Rahway, N. J., crystalline DL-thyroxine from E. R. Squibb and Sons, New Brunswick, N. J., and methionine from the U. S. Industrial Chemical Co., New York, N. Y.

LITERATURE CITED

BEITHEL, J. J., AND H. A. LARDY 1948 Comparative effectiveness of vitamin B_{12}, whole liver substance and extracts high in APA activity, as growth promoting materials for hyperthyroid animals. J. Nutrition, *37:* 495.

EMERSON, G. A. 1949 Growth promoting activity of vitamin B_{12} in rats receiving thyroid substance. Proc. Soc. Exp. Biol. Med., *70:* 392.

NICHOL, C. A., A. R. ROBBLEE, W. W. CRAVENS AND C. A. ELVEHJEM 1947 Activity of antipernicious anemia preparations in chicks. J. Biol. Chem., *170:* 419.

REGISTER, U. D., W. R. RUEGAMER AND C. A. ELVEHJEM 1949 An improved assay for a growth factor in liver extracts. Ibid., *177:* 129.

SURE, B. 1941 The existence of a new dietary factor essential for lactation. J. Nutrition, *22:* 499.

NUTRITIONAL VALUE OF PLANT MATERIALS

III. ACUTE UREMIA OF NEWBORN RATS FROM MOTHERS FED PLANT RATIONS [1]

HARLYN O. HALVORSON AND M. O. SCHULTZE
Division of Agricultural Biochemistry, University of Minnesota, St. Paul

(Received for publication May 29, 1950)

When rats were raised from weaning on purified rations in which a commercial soybean proteinate [2] and DL-methionine furnished the only source of amino acids, their offspring were found to have a high incidence of early mortality (Schultze, '49a). A very high concentration of urea in the blood was found to precede the death of the afflicted young (Schultze and Halvorson, '49), and this condition has therefore been referred to as "acute uremia of the newborn" (Schultze, '49b). The essential features of the external symptomatology of acute uremia of the newborn and its prevention by a single subcutaneous injection of 0.05 μg of vitamin B_{12} into the newborn rat have recently been described (Schultze, '49b).

The present paper presents evidence dealing with: (a) the incidence of the syndrome in rats born to mothers maintained on rations composed mainly of crude or purified plant ingredients; (b) the preventive effect of some substances of animal origin; and (c) the changes in the blood urea concentration of very young rats.

[1] Paper no. 2519, Scientific Journal Series, Minnesota Agricultural Experiment Station.

[2] Archer-Daniels-Midland Co., Minneapolis.

EXPERIMENTAL

Animals and rations

The rats used were mainly of a black strain of the "Line 3" maintained in these laboratories for many years. A few rats from a closely related albino strain were included. Since a difference in the performance of the two strains was not evident, the results from both strains were pooled. The females were bred when they were at least 10 weeks old or, in the case of the slow-growing rats fed the corn-soybean-alfalfa rations, when they weighed at least 150 gm. They were returned to the breeding cages immediately after weaning or after the loss of a litter. All rats were kept on raised wire screens except shortly before parturition and during the nursing period, when they were maintained on clean wood shavings.

The purified rations, which contained a commercial or a purified soybean protein,[3] DL-methionine, hydrogenated vegetable fat, corn oil, salts and all available vitamins except ascorbic acid and vitamin B_{12}, have been described in detail in another publication (Schultze, '50). These rations were designated as S_1, S_2 and S_3, respectively. Acute uremia of the newborn was also observed in young rats born to mothers maintained on rations designated as CS-1, CS-2 and CS-3, which were composed of yellow corn, alfalfa meal and expeller-processed soybean meal. The composition of these rations is given in table 1. Rations CS-1 and CS-2 have been previously described by Spitzer and Phillips ('46). Ration CS-3 was included to provide a higher protein content. Due to the poor growth performance of the rats, the CS rations were supplemented in one series of experiments with 0.50% of DL-methionine and the mixture of choline and B vitamins, including biotin, described elsewhere (Schultze, '50).

[3] The term "purified protein" as used in this paper refers to the mixture of soybean proteins remaining after two reprecipitations from aqueous solution and extensive extraction with boiling alcohol. It is recognized that soybeans contain several proteins (Briggs and Mann, '50). The method of purification used for our product was not intended to effect separation of the different soybean proteins.

Determination of blood urea

For routine determinations of the urea concentrations in the small volumes of blood available from individual young rats, the colorimetric procedure of Archibald ('45) appeared to be most suitable. Since this method is not absolutely specific for urea, studies on its specificity and reproducibility when applied to normal and uremic rats' blood were carried out. As is shown by the data presented in another paper (Halvorson and Schultze, '50), the condition described here is a true uremia. In uremic blood the non-urea chromogens

TABLE 1

Composition of natural rations

CONSTITUENTS	RATION		
	CS-1	CS-2	CS-3
	gm	*gm*	*gm*
Ground yellow corn	753.4	653.4	553.4
Expeller-processed soybean meal	175.0	175.0	375.0
Alfalfa meal	50.0	150.0	50.0
Salt mixture [1]	21.6	21.6	21.6

[1] Salt mixture: $CaHPO_4$ 10.0 gm; $CaCO_3$ 6.5 gm; NaCl, iodized, 5.0 gm; $MnSO_4$ 0.1 gm. Vitamins A and D were supplied as two drops of halibut liver oil (Abbott) per rat per week (Spitzer and Phillips, '46).

do not account for more than about 12% of the color measured and reported here as urea. The blood was collected and analyzed as described elsewhere (Halvorson and Schultze, '50).

RESULTS AND DISCUSSION

Incidence of acute uremia of the newborn

Under the conditions of these experiments, the external symptoms of acute uremia of the newborn usually appear in 36 to 48 hours after birth. Inasmuch as death often occurs within 6 hours after the appearance of the first symptoms, and since not all litters or all young within a litter are af-

fected, it is difficult to obtain a precise evaluation of the incidence of the syndrome. For this reason the incidence is expressed in terms of per cent of litters in which one or more of the young were actually observed in the crisis of acute uremia of the newborn. As is shown in table 2, from 40 to 50% of the litters born to females maintained on the unsupplemented rations containing the commercial soybean protein incurred symptoms of acute fatal uremia. Among the rats listed in groups 14 to 22 in table 2 which were fed the natural rations the incidence of acute uremia appeared to be slightly lower. This may be due in part to the fact that many of the young died before they were 36 to 48 hours old. In view of the high mortality rate of the young born to rats maintained on the unsupplemented CS rations, the number of F_1 generation offspring which could be raised to the reproductive stage was too small to permit a separate evaluation of the incidence of uremia among the parent and F_1 generations.

The data summarized in table 2 indicate that the incidence of acute uremia does not increase with successive generations maintained on the same ration (groups 1–4). The addition of biotin to the vitamin supplement (ration S_2, group 11) had no effect on the incidence of uremia. The failure to observe the typical symptoms of acute uremia of the newborn among the rats of group 12 maintained on the highly purified soybean protein cannot be satisfactorily explained at present. The method of purification of the commercial soybean protein, described in detail elsewhere (Schultze, '50), is such that the vitamin B_{12} activity present in the starting material should be extracted or destroyed. If rats are maintained on ration S_3 containing this purified protein for sufficiently long periods or for more than one generation, about 90% of their offspring die, without, however, showing the typical symptoms of the acute uremia of the newborn previously described.

Prevention of acute uremia of the newborn

The data presented in table 2 show clearly that a mixture of DL-methionine, choline or the B vitamins (except vitamin

TABLE 2

Incidence of acute uremia of the newborn

(Per cent of litters in which one or more young were observed with acute uremia)

	GROUP	RATION	SOURCE OF PROTEIN	SUPPLEMENT	GENERATION OF RATS ON PLANT RATION	TOTAL NO. OF LITTERS OBSERVED	% LITTERS WITH ACUTE UREMIA
1		S_1	Commercial soy protein	None	Parent	80	50.0
2		S_1	Commercial soy protein	None	F_1	128	46.9
3		S_1	Commercial soy protein	None	F_2	50	44.0
4		S_1	Commercial soy protein	None	F_3	40	45.0
5	Litter mates	S_1	Commercial soy protein	None	F_1	16	56.2
6	Litter mates	S_1	Commercial soy protein	2% liver extract	F_1	20	0
7	The same rats selected from groups 1 and 2	S_1	Commercial soy protein	None	Parent and F_1	37	84.0
8	The same rats selected from groups 1 and 2	S_1	Commercial soy protein	2% liver extract	Parent and F_1	17	0
9	Litter mates	S_1	Commercial soy protein	None	F_1	40	37.5
10	Litter mates	S_1	Commercial soy protein	3% condensed fish solubles	F_1	44	0
11		S_2	Commercial soy protein	None	F_1 and F_2	61	49.2
12		S_3	Purified soy protein	None	F_2 and F_3	61	0
13		C_1	Purified casein	None	..	289	0
14		CS-1	Corn, soybean meal, alfalfa	None	Parent and F_1	69	40.6
15		CS-1	Corn, soybean meal, alfalfa	Methionine, choline, B vitamins [1]	Parent and F_1	25	40.0
16		CS-1	Corn, soybean meal, alfalfa	3% condensed fish solubles	F_1	14	0
17		CS-2	Corn, soybean meal, alfalfa	None	Parent and F_1	56	37.5
18		CS-2	Corn, soybean meal, alfalfa	Methionine, choline, B vitamins [1]	Parent and F_1	34	29.4
19		CS-2	Corn, soybean meal, alfalfa	3% condensed fish solubles	F_1	21	0
20		CS-3	Corn, soybean meal, alfalfa	None	Parent and F_1	55	30.9
21		CS-3	Corn, soybean meal, alfalfa	Methionine, choline, B vitamins [1]	Parent and F_1	38	29.0
22		CS-3	Corn, soybean meal, alfalfa	3% condensed fish solubles	F_1	15	0

[1] The vitamin-sucrose mixture described by Schultze ('50) at a 2% level and 0.50% of DL-methionine.

B_{12}) in the amounts furnished was not able to prevent the establishment of the syndrome. It should be borne in mind that the rations fed to groups 1 through 13 (table 2) contained the same mixture of methionine, choline and B vitamins which was added for groups 15, 18 and 21 to the quantities of these compounds naturally contained in the corn, soybean meal and alfalfa. In view of the previously reported observation (Schultze, '49b) that subcutaneous injection of vitamin B_{12} into the newborn rat can prevent the uremic syndrome, the protective effect of liver extract 1:20 [4] and of condensed fish solubles is presumably due to the vitamin B_{12} activity of these substances. This protective effect is particularly well illustrated in group 8, in which the 14 rats from group 7 whose litters had a very high incidence of acute uremia were subsequently transferred as group 8 to ration S_1 supplemented with 2% liver extract. Their next litters did not show the acute uremia. In the etiology of the syndrome maternal nutrition plays, therefore, a decisive role. The defect in maternal nutrition can be corrected by subsequent proper feeding.

The fact that on the ration containing 24% purified casein (crude lactic casein extracted in a percolator for 72 hours with 95% alcohol at 55 to 60°C.) no acute uremia was observed indicates a highly protective effect of cows' milk on this syndrome. The rats in group 13 which were fed the casein ration were used in connection with other experiments. Careful observations made after parturition permit their inclusion in this paper. Using the growth of rats as a criterion of assay, Hartman et al. ('49) have classified alfalfa among those substances which possess "nutrient X or vitamin B_{12}" activity, whereas Lewis et al. ('49) found alfalfa leaf meal to contain no significant amount of vitamin B_{12} activity. On the basis of a microbiological assay with *Lactobacillus leichmannii* (American Type Culture Collection 4797), Peeler et al. ('49) reported that yellow corn, soybean meal and alfalfa leaf meal contained 10, 11 and 40 mμg of vitamin B_{12} per gram. In the

[4] Wilson.

experiments, the inclusion of as much as 15% of alfalfa meal present in the ration failed to prevent the occurrence of acute uremia of the newborn.

Concentration of urea in the blood of very young rats

All data on blood urea presented in this paper are based on the method of Archibald ('45). While these values are referred to as "blood urea," it is recognized that they include a small and perhaps variable fraction of compounds other than urea. This limitation of analytical methodology does not invalidate the conclusions which can be drawn from the data in table 3 that: (1) shortly after birth and after the first nourishment is obtained, the blood urea concentration of normal-appearing young rats increases, to reach a maximum value generally in about 48 to 72 hours after birth; (2) this transient rise in blood urea is not prevented by such substances in the diet of the mother as can prevent the appearance of external symptoms of acute uremia of the newborn; (3) after 24 hours from birth the blood urea concentration may rise very rapidly to over 200 mg per 100 ml in those individuals that show the typical external symptoms of an acute uremic crisis; (4) during the first few days after birth the urea concentration in the blood of young rats shows great variations, as is indicated by the high standard deviations; (5) normal-appearing very young rats may have blood urea values up to 120 mg per 100 ml in about 24 to 48 hours after birth. These animals might have shown symptoms of acute uremia within a few hours if they had not been sacrificed for analytical purposes.

The present data suggest that young rats born to mothers maintained on certain purified or natural rations composed of plant ingredients suffer from a defect in nitrogen metabolism which results in an increase of the urea concentration in the blood. Inasmuch as the uremia develops only after the young have been nursed by their mothers, and since their consumption of milk in all cases was abundant, it appears that the uremia is associated with the metabolism of absorbed

TABLE 3

Mean blood urea concentration of young rats born to mothers fed various rations

The number of rats from which the means were calculated is given in parenthesis

RATION OF MOTHERS	RATS WITH NORMAL APPEARANCE					RATS WITH SYMPTOMS OF ACUTE UREMIA	
	S_1	S_1 plus 3% fish solubles	S_1 plus 2% liver extract	CS-1, 2 and 3	CS-1, 2 and 3 plus 3% fish solubles	S_1	CS-1, 2 and 3
Age	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
0 hours	38.8 ± 7.2 [1](14)			32.4 ± 5.9 [1](12)	33.2 ± 3.3 [1](8)		
½–6 hours [2]	49.2 ± 7.9 (13)	43.9 ± 8.1 [1](7)		47.7 ± 9.0 (14)	52.0 ± 16.1 (10)		
6–12 hours	43.3 ± 16.8 (6)	55.5 ± 15.2 (8)		54.4 ± 13.8 (12)	55.0 ± 12.0 (10)		
12–24 hours	59.5 ± 21.0 (24)		37.5 ± 13.4 [1](6)	92.7 ± 45.9 (13)	46.1 ± 8.5 (10)		
24–48 hours	70.8 ± 29.1 (28)	58.5 ± 22.5 (14)	43.3 ± 9.1 (5)	118.8 ± 35.0 (13)	40.0 ± 15.0 (15)	145.6 ± 41.8 [1](24)	196.3 ± 38.7 [1](15)
48–72 hours	64.5 ± 30.0 (27)	50.3 ± 28.9 (14)	49.9 ± 20.7 (7)			220.2 ± 60.0 (11)	
72–96 hours	61.7 ± 14.6 (9)	55.9 ± 15.2 (17)	59.3 ± 21.0 (5)				
96–120 hours	63.2 ± 18.9 (18)	57.2 ± 19.7 (14)					
6 days	53.1 ± 12.1 (16)	54.2 ± 21.4 (9)					
7 days	58.0 ± 27.0 (12)	44.6 ± 16.6 (8)					
8 days	48.7 ± 17.7 (11)	48.6 ± 16.7 (8)					
21 days	28.0 ± 8.0 (13)						

[1] Standard deviation.

[2] These animals had been nursed by their mothers, while those taken at zero hours had not.

nutrients. In some individuals the accumulation of urea in the blood becomes so great that a typical syndrome of acute uremia develops. Data reported elsewhere (Halvorson and Schultze, '50), as well as other studies made in this laboratory, indicate that under these conditions there is also a marked increase of the non-protein constituents of the blood other than urea.

If the maternal organism is supplied with substances which are good sources of vitamin B_{12} activity, the blood urea concentration remains at a lower level. In line with observations previously reported from this laboratory, the development of symptoms of acute uremia in rats born to mothers on natural unsupplemented plant rations (CS-1, 2 and 3) can be prevented by subcutaneous injection of a single dose of 0.05 μg of vitamin B_{12} [5] shortly after birth. Thus, from 35 newborn rats injected with vitamin B_{12} none was observed with acute uremia, while in 12 of 42 uninjected litter mates typical symptoms of acute uremia were noticed prior to their death.

With regard to the etiology of acute uremia of the newborn, the pathological involvement of the kidney cannot be dismissed from consideration. Elevated non-protein nitrogen values in the blood of weanling rats with renal damage have been reported in several instances (Griffith, '41; Zucker and Zucker, '48; Olson and Deane, '49). The fact that at birth the blood urea concentration is in the normal range, and that the uremia can be readily prevented by administration of vitamin B_{12} after birth, seems to suggest, however, that the acute uremia of the newborn is primarily of metabolic origin.

On all the rations young rats were occasionally observed which became unthrifty and weak in about a week or 10 days after birth. In several instances blood urea concentrations of up to 200 mg per 100 ml were found in such animals. This condition appears to be a chronic form of uremia in which the animals survive for one to two weeks without gaining much weight. The prolonged survival differentiates this con-

[5] Cobione, Merck and Co.

dition from the acute uremia of the newborn, in which the young seldom survive as long as 60 hours after birth.

Very few data appear to be available in the literature which might be used for comparison with the values for blood urea recorded here. From the data of Daly et al. ('47), who determined the blood urea nitrogen in 9 specimens of the pooled blood of fetuses removed from rats shortly before parturition, a mean concentration of blood urea of 35.9 mg per 100 ml was calculated. Heller ('49a) found that the plasma urea of 24-hour-old normal rats was 11.1 milli-equivalents per liter of water, which corresponds to about 63.4 mg of urea per 100 ml of blood plasma. The mothers of these rats were maintained on a ration containing meat, milk, and fish proteins (Heller, '49b).

SUMMARY

1. Young rats born to mothers maintained on rations in which a commercial soybean protein preparation and DL-methionine supplied the only source of amino acids developed an acute fatal uremia in about 50% of the litters.

2. Within 48 hours after birth the blood urea concentration may rise to over 200 mg per 100 ml of blood. The rise in blood urea of the afflicted young was particularly rapid in 24 to 48 hours after birth.

3. In those individuals which did not show symptoms of acute uremia there was a transient rise in the concentration of blood urea which reached a maximum in about 48 to 72 hours after birth.

4. Acute uremia was also observed in about 40% of the litters born to rats maintained on rations composed of yellow corn, soybean meal and alfalfa meal.

5. The acute uremia of the newborn could be prevented by the feeding of alcohol-extracted casein, condensed fish solubles, or liver extract to the mothers or by subcutaneous injection of vitamin B_{12} into the young shortly after birth.

ACKNOWLEDGMENTS

The assistance of Merck and Co., the Abbott Laboratories, the Wilson Laboratories, the Lederle Laboratories, Du Pont

and Co., and the Archer-Daniels-Midland Co. with various supplies is gratefully acknowledged. Mr. Harold Segal aided with the purification of the soybean protein.

LITERATURE CITED

ARCHIBALD, R. M. 1945 Colorimetric determination of urea. J. Biol. Chem., *157:* 507.

BRIGGS, D. R., AND R. L. MANN 1950 An electrophoretic analysis of soybean protein. Cereal Chem., *27:* 243.

DALY, H., L. J. WELLS AND G. EVANS 1947 Experimental evidence of the secretion of urine by the fetal kidney. Proc. Soc. Exp. Biol. Med., *64:* 78.

GRIFFITH, W. H. 1941 The relation of choline to the kidneys. Biological Symposia, *5:* 193.

HALVORSON, HARLYN O., AND M. O. SCHULTZE 1950 Studies on the colorimetric determination of urea in the blood of normal and uremic rats. J. Biol. Chem., in press.

HARTMAN, A. M., L. P. DRYDEN AND C. A. CARY 1949 The role and sources of vitamin B_{12}. J. Am. Diet. Assn., *25:* 929.

HELLER, H. 1949a Effects of dehydration on adult and newborn rats. J. Physiol., *108:* 303.

———— 1949b Personal communication.

LEWIS, U. J., U. D. REGISTER, H. T. THOMPSON AND C. A. ELVEHJEM 1949 Distribution of vitamin B_{12} in natural materials. Proc. Soc. Exp. Biol. Med., *72:* 479.

OLSON, R. E., AND H. W. DEANE 1949 A physiological and cytochemical study of the kidney and the adrenal cortex during acute choline deficiency in weanling rats. J. Nutrition, *39:* 31.

PEELER, H. T., H. YACOWITZ AND L. C. NORRIS 1949 A microbiological assay for vitamin B_{12} using *Lactobacillus leichmannii*. Proc. Soc. Exp. Biol. Med., *72:* 515.

SCHULTZE, M. O. 1949a Growth and reproduction of rats on rations devoid of animal protein. Fed. Proc., *8:* 395.

———— 1949b Nutritional value of plant materials. II. Prevention of acute uremia of the newborn rat by vitamin B_{12}. Proc. Soc. Exp. Biol. Med., *72:* 613.

———— 1950 Nutritional value of plant materials. I. Growth of rats on purified rations containing soybean protein. J. Nutrition, *41:* 103.

SCHULTZE, M. O., AND HARLYN O. HALVORSON 1949 Acute uremia of suckling rats born to mothers on rations devoid of animal protein. J. Animal Sci., *8:* 629.

SPITZER, R. R., AND P. H. PHILLIPS 1946 Reproduction and lactation studies with rats fed natural rations. J. Nutrition, *32:* 631.

ZUCKER, L. M., AND T. F. ZUCKER 1948 Zoopherin: a nutritional factor for rats associated with animal protein sources. Arch. Biochem., *16:* 115.

STUDIES ON THE COMPARATIVE NUTRITIVE VALUE OF FATS

XIII. GROWTH AND REPRODUCTION OVER 25 GENERATIONS ON SHERMAN DIET B WHERE BUTTERFAT WAS REPLACED BY MARGARINE FAT, INCLUDING A STUDY OF CALCIUM METABOLISM[1,2]

HARRY J. DEUEL, JR., SAMUEL M. GREENBERG AND EVELYN E. SAVAGE

Department of Biochemistry and Nutrition, University of Southern California, Los Angeles

AND

LUCIEN A. BAVETTA[3]

School of Dentistry, University of Southern California, Los Angeles

ONE FIGURE

(Received for publication May 31, 1950)

In an earlier report from this laboratory (Deuel et al., '45) it was shown that rats continued in excellent nutritional condition over 10 generations when they were fed on Sherman diet B, modified by replacing the whole milk powder with skimmed milk powder plus a proportionate amount of a hydrogenated vegetable margarine fat. The growth rate of successive generations of the rats was shown to increase progressively during the period of the test and it had reached

[1] This work was done under a research grant from The Best Foods, Inc. The authors wish to acknowledge the helpful advice of Professor Anton J. Carlson of the University of Chicago, of Professor Arthur W. Thomas of Columbia University, and of Dr. H. W. Vahlteich of The Best Foods, Inc., during the course of the experiments.

[2] Contribution no. 258 of the Department of Biochemistry and Nutrition, University of Southern California.

[3] The data on calcium content and on calcium balances were reported by Professor L. A. Bavetta at the meeting of the Southern California State Dental Association in Los Angeles in August, 1949.

a maximum level in the 10th generation. Moreover, pregnancy and lactation continued satisfactorily throughout the experiment. The fertility of the 10th generation was excellent, since 13 of 14 females bred and cast litters, of which only two litters were destroyed before three days or were stillborn. No rats died in the remaining 11 litters and the average 21-day weight of the 75 rats weaned was as great or greater than that of any other previous generation except the 9th one.

Sherman and Campbell ('24) reported earlier that their rats grew better and were larger at all ages on the "B" diet where whole milk powder comprised one-third of the total weight than on the "A" diet in which only one-sixth of the total diet was whole milk powder. It was later shown that the life span of the rats on the B diet had been increased by a significant amount (Sherman and Campbell, '29–'30). In the most recent report (Sherman and Trupp, '49), the experiment with diet A had progressed to the 70th generation.

Although it is evident that a diet which will support growth, reproduction and lactation satisfactorily over 10 generations is, in all probability, a nutritionally adequate one, it was of importance to determine whether the high level of growth and reproduction attained could be maintained over an extended period. Since both the original Sherman diet B as used at Columbia University, and the modified Sherman diet on which studies have been made at the University of Southern California, have proved successful in multigeneration tests, it seemed worthwhile to compare growth, longevity, reproduction, lactation, calcium content, and calcium balances in rats maintained on these two diets. The present paper reports data on the growth of the 11th to the 24th generations and on reproductive and lactation performances from the 11th to the 25th generation. Experiments are also reported for a group where the lineage was continued through the second litter rats. Data comparing growth, reproduction and the calcium content of rats from the original Sherman diet and the modified Sherman diet are included.

EXPERIMENTAL

The procedures employed in these tests were identical with those reported in our earlier paper (Deuel et al., '45). The rats were maintained on a diet containing ground whole wheat (66.00%), sodium chloride (1.0%), skimmed milk powder (23.76%) and margarine fat (9.24%). This diet is identical with the Sherman diet B except that margarine fat and skimmed milk powder replace the whole milk powder. The rats were supplemented once weekly with 5 gm of ground lean horse meat, except that the female rats did not receive this supplement during the lactation period. The animals were further supplemented with 5 gm of lettuce each, once weekly. Such supplementation is also employed by Sherman in his multigeneration experiments.

The young rats were weaned at 21 days of age and were then housed in large cages bedded with pine shavings, with 4 rats to a cage. Food was given ad libitum and water was constantly available. The animals were maintained in a thermostatically controlled room. Food was prepared fresh twice weekly and the reserve stock was kept in a refrigerator. The animals were weighed weekly.

The rats were bred at 13 weeks of age, using one male to two female rats. Where both females in a single cage failed to become pregnant in 6 weeks, they were subsequently mated with a male of proved fertility. Litter mates were never bred. Litters were weighed at three days, at which time they were reduced to 7 rats.

In addition to the main group of rats, where the lineage was through the first litter, an additional series was carried out in which the second litter of the second litter of each generation was used. The original "second litter" series was dropped after the 12th generation because of lack of significance with respect to comparisons between this series and the "first litter" series. However, a new "second litter" series was started from the 15th generation rats which has been carried through the 22nd generation. This series was

reestablished because of the poor performance of the "first litter" group of the 15th generation.

It seemed desirable to compare the growth and reproduction of the rats on the modified Sherman diet with those of animals receiving the original Sherman B diet. Since no animals of similar strain were available which had received the Sherman diet B over a comparable number of generations, young adult rats from our own colony which had previously received our stock diet were transferred to the Sherman diet B, bred, and the progeny used for comparison with the progeny of the 20th generation of rats which had continuously received the modified Sherman diet. Further comparisons between these diets were effected by subdividing each main group into two subgroups, which were fed either the original Sherman diet B or the modified Sherman diet. Studies were made on growth, reproduction and bone length of rats started on their respective diets at 21 days of age. Analyses for the ash and calcium content of 21-day-old and mature rats were carried out on the first litter progeny of the rats on the above experiment. Calcium balance studies were made on the mature rats just prior to their sacrifice for mineral analysis.

The bone length was determined by a modification of our earlier procedure, described elsewhere (Greenberg et al., '50). In the case of the 21-day-old rats, calcium was estimated on the whole animal. The rats were fasted for 24 hours and killed by ether anesthetization, the gastrointestinal tracts were removed and washed out, and the animals with the cleaned tracts were ashed. The calcium content of the ash was obtained by the usual oxalate-permanganate method. In the case of the older rats, calcium was determined on an aliquot of the hashed tissue of the rats, from which the gastrointestinal tract had previously been removed.

RESULTS AND DISCUSSION

The rate of growth of representative generations from the first to the 24th is given in figure 1, while the reproduction

TABLE 1

Reproduction and lactation performances of first litter rats from the 11th to the 25th generation and of second litter rats from the 16th to the 22nd generation receiving the modified Sherman diet B in which a hydrogenated vegetable margarine fat and skimmed milk powder replaced whole milk powder

PARENTS			LITTER					
Generation	Females bred	Litters born	Average period after mating [1]	Generation	Average number per litter	Average weight/rat		Number of litters destroyed or stillborn [3]	Young not surviving 3–21 day period
						At 3 days	At 21 days [2]		
			days			*gm*	*gm*		
					First litter rats				
11	16	13	42.3	12	8.4	7.9	36.6 (63)	2	0
12	13	12	36.3	13	6.9	7.4	33.5 (56)	0	0
13	18	8	27.2	14	6.2	8.5	34.5 (28)	1	3
14	4	2	53.0	15	7.0	7.0	26.4 (7)	1	0
15	2	2	30.0	16	8.5	6.8	32.3 (14)	0	0
16	8	8	24.4	17	9.8	7.1	38.9 (35)	1	0
17	14	12	31.2	18	6.6	6.6	35.5 (21)	7	0
18	15	14	26.4	19	8.2	7.5	32.8 (28)	4	8
19	26	18	28.1	20	7.3	7.5	35.2 (70)	2	2
20	27	15	28.0	21	8.2	7.4	36.3 (77)	1	1
21	18	16	25.8	22	8.1	7.6	33.9 (49)	0	18
22	27	23	28.3	23	7.5	7.2	33.6 (91)	0	2
23	27	27	31.8	24	7.2	7.8	32.8 (84)	5	4
24	23	22	26.1	25	9.8	7.8	33.1 (112)	1	1
25	28	27	27.2	26	11.1	7.3	34.1 (119)	3	7
					Second litter rats				
16	6	6	32.3	17	10.5	7.1	40.3 (35)	0	2
17	18	14	27.4	18	6.4	7.3	33.9 (14)	7	1
18	14	14	28.3	19	9.1	8.6	39.5 (77)	2	1
19	17	15	26.1	20	7.9	8.6	39.0 (49)	2	10
20	27	22	29.8	21	6.9	6.9	37.1 (63)	3	5
21	18	14	26.2	22	7.5	7.5	36.8 (56)	0	4

[1] Time after male was placed in cage with females.

[2] Only rats in litters of 7 are included in averages. All litters larger than 7 were reduced to that number at three days of age. The figures in parentheses represent the number of rats included in the mean.

[3] Not found, or dead, three days after birth.

and lactation performances of individual generations are summarized in table 1. The latter data include the results of the "first litter" series only from the 11th through the 25th generation and for the new "second litter" group from the 15th through the 22nd generation. Earlier results are reported in our first paper (Deuel et al., '45).

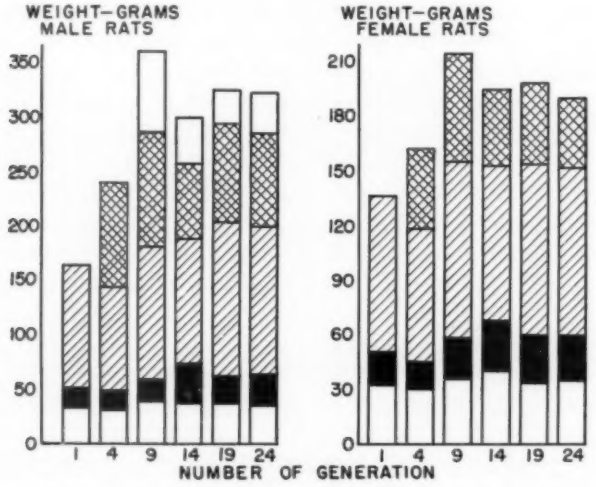


Fig. 1 The body weight increments at 21, 30, 60, 90 and 120 days of age of representative successive generations of male and female rats which received the modified Sherman diet. Body weights at 21 days are shown by lower blank areas, those at 30 days to top of black area, those at 60 days to top of hatched area, those at 90 days to top of cross-hatched area, and those at 120 days to top of upper blank space.

Although data on growth are reported in figure 1 only for every 5th generation, these are unselected and represent average growth responses. In the case of the male rats at 80 days of age, for which data are complete throughout the 25 generations, the maximum average weight was reached by the 10th generation (310 gm) and the minimum average weight by the second generation (192 gm), compared with the 80-day weight of the first generation of 227 gm. The

average 80-day weights of the 21st to 25th generations, inclusive, were 238, 243, 235, 265 and 239 gm, respectively. The weights of male rats were recorded at 120 days only after the 7th generation. The maximum average at this period was 398 gm in the 10th generation, and the minimum was 274 gm in the 15th generation. The mean values for the 21st to 25th generations were 293, 308, 298, 321 and 293 gm, respectively.

Since the female rats were bred after 90 days, valid comparisons are not available at later periods. However, at 90 days a minimum average weight was obtained in the second generation (166 gm) and a maximum in the 10th generation (216 gm). The mean body weights for the 21st to 25th generations were 190, 190, 181, 190 and 188 gm, respectively.

The fertility of the rats was maintained throughout the test. From the start through the 5th generation, 100% of the females bred and cast litters. A minimum response was obtained in the 13th generation, in which only 44% (8 of 18) were fertile. The results for the last 5 generations were excellent; namely 89% (16 of 18), 85% (23 of 27), 100% (27 of 27), 95% (22 of 23) and 96% (27 of 28), respectively. Only two females were available for breeding in the 15th generation but these both cast litters. The poor showing of this generation is probably to be ascribed to the effect of an excessive application of DDT, used to control pests. The treatment with DDT was discontinued after the 15th generation. The 16th generation showed a prompt recovery in growth and reproduction, and toxic symptoms have not been noted in subsequent generations. Mites and lice were controlled thereafter by the use of minimum quantities of naphthalene flakes placed in the fresh bedding.

The lactation performance as judged by the weaning weights (21 days) of the rats has remained fairly constant. The average body weights of 7 rat litters was 31.1 gm (35 rats) in the beginning stock rats. The minimum mean weight of 26.4 gm (7 rats) was obtained with the 14th generation

mothers, while the maximum value of 39.3 gm (35 rats) was noted for the 7th generation mothers. The performance of the 21st to the 25th generation mothers has been good; namely 33.9 gm (49 rats), 33.6 gm (91 rats), 32.8 gm (84 rats), 33.1 gm (112 rats) and 34.1 gm (119 rats) for the average 21-day weights of the progeny.

In the case of the new series of "second litter" rats, somewhat better growth was reached at 120 days than was noted with the corresponding "first litter" series. Thus, the average values for male rats from the 17th to the 21st generations at this period were 356, 336, 342, 348 and 378 gm, respectively. The average 120-day weight of the last 5 generations of "second litter" series was 352 gm, compared with a value of 303 gm for the last 5 litters of our "first litter" series, which were run concurrently. The corresponding averages for the females at 90 days were 207 and 188 gm for the "second litter" and "first litter" series, respectively.

From the standpoint of fertility, the percentage of sucessful first pregnancies of the "second litter" series was somewhat lower than that of the "first litter" group. The proportion of females which cast a litter on the first breeding was as follows for the 17th through the 21st generations, inclusive: 84% (21 of 25), 75% (15 of 20), 91% (20 of 22), 87% (27 of 31) and 81% (22 of 27), respectively. The reproductive performances were not strictly comparable on the second breedings, since some rats, including the females sterile in the first series, were excluded. The values for successful pregnancies in the second breeding for the 17th to 21st generations, respectively, were 78% (14 of 18), 100% (14 of 14), 88% (15 of 17), 81% (22 of 27) and 78% (14 of 18). The lactation of the "second litter" mothers during their second litter, as indicated by the weaning weight of the offspring, appeared to be somewhat better than that of the "first litter" series or than that of the first litter of the "second litter" series. Thus, the average 21-day weights were 33.9 gm (14 rats), 39.5 gm (77 rats), 39.0 gm (49 rats), 37.1 gm (63 rats) and 36.8 gm (56 rats) for the second litters

of the 17th to the 21st generation of the "second litter" series, respectively.

A comparison of the growth, reproductive capacity and longevity of the 21st generation rats with those of rats born of stock animals which had been placed on Sherman diet B at breeding is reported in tables 2 and 3. Groups M and N are composed of progeny of stock rats placed on Sherman diet B at breeding. Group M was continued on Sherman diet B from weaning, while group N was assigned to the modified Sherman diet at the same time. Groups O and P were from the 20th generation of rats on the modified Sherman diet. Group O was transferred to the original Sherman diet B at weaning while group P was continued on the modified Sherman diet.

No differences obtained in the rate of growth of the females irrespective of previous dietary history or the diet to which they were assigned. In the case of the male rats, a slightly slower rate of growth resulted in group P, although they eventually reached approximately the same size as those in the other groups. On the other hand, group N, which also received the modified Sherman diet, grew somewhat better than the other groups during the early part of the test and at an equal rate during the latter part. The slightly lower rate of growth of group P may possibly be ascribed to the fact that this group had the lowest body weights at weaning. The data on growth reported in table 2 are borne out by the tibia lengths recorded in table 3.

There was no appreciable difference in survival at one year so far as diet was concerned. Of the original 40 rats from each original dietary regime, 36 rats were still alive from the original Sherman group (groups M and N) and 37 from the modified Sherman animals (groups O and P) after one year. When the comparison is based on the current dietary regime, irrespective of previous dietary history, the survivals at one year were 38 (groups M and O) and 35 (groups N and P) animals, respectively. At the end of 590 days, when the experiments were terminated, 30 (groups M

TABLE 2

The body weights and survival of male and female rats previously on Sherman diet B (1) or modified Sherman diet (II) and distributed between these diets at weaning. Ten rats per group at start

GROUP	DIET		AVERAGE 21-DAY BODY WEIGHT	AVERAGE BODY WEIGHTS AT FOLLOWING AGES IN DAYS [1]											
	Previous maternal diet	Current		50	100	150	200	250	300	350	400	450	500	550	590
			gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
Male rats															
M	I	I	41	156	298	351	395	413	406	428	450	444	460	463 (9)	442 (9)
N	I	II	42	170	317	369	407	420	429	440	453	441	465 (8)	452 (8)	428 (7)
O	II	I	39	152	291	352	409 (9)	430 (9)	418 (9)	442 (9)	451 (9)	468 (8)	448 (8)	452 (7)	413 (7)
P	II	II	37	136	274	310	364 (9)	388 (9)	393 (9)	414 (8)	424 (8)	425 (8)	417 (7)	455 (6)	445 (6)
Female rats — Average age				50	90	170	200	340	380	520	550	590			
M	I	I	39	124	188 (9)	229 (9)	237 (9)	264 (9)	274 (9)	302 (9)	302 (9)	303 (9)			
N	I	II	40	132	189 (9)	227 (9)	246 (8)	264 (7)	274 (7)	318 (6)	322 (5)	325 (5)			
O	II	I	40	132	194	237	252	272	277	335 (6)	338 (6)	341 (5)			
P	II	II	40	128	194	236	240	261	275	310 (7)	314 (7)	300 (7)			

[1] The values in parentheses indicate the number of animals surviving at this period.

TABLE 3

Body weights and tibia lengths as determined by the x-ray of male and female rats previously on Sherman diet B (I) or on modified Sherman diet (II) and distributed between these diets. Ten rats in each group unless other number indicated in parentheses

GROUP	DIET		BODY WT. IN GM AT FOLLOWING AGES IN DAYS [1]				TIBIA LENGTHS IN CM AT FOLLOWING AGES IN DAYS [1]			GAIN IN BONE LENGTH IN CM [2]	
	Previous maternal diet	Current	21	90	180	365	90	180	365	90–180	90–365
						Male rats					
M	I	I	41 ± 1.4	278 ± 10.5	378 ± 13.8	434 ± 12.3	3.79 ± 0.06 (9)	4.14 ± 0.05 (9)	4.19 ± 0.05 (8)	0.35	0.40
N	I	II	42 ± 1.6	301 ± 5.7	396 ± 9.4	446 ± 13.7	3.79 ± 0.03 (9)	4.17 ± 0.02 (9)	4.19 ± 0.04 (8)	0.38	0.40
O	II	I	39 ± 2.3	273 ± 10.5	375 ± 16.2	451 ± 14.7 (9)	3.69 ± 0.04 (8)	4.02 ± 0.05 (8)	4.18 ± 0.03 (8)	0.33	0.49
P	II	II	37 ± 2.1	254 ± 11.1	354 ± 14.2 (9)	420 ± 14.8 (8)	3.52 ± 0.05 (8)	3.88 ± 0.04 (8)	3.98 ± 0.02 (7)	0.36	0.46
						Female rats					
M	I	I	39 ± 1.4	188 ± 6.2 (9)	233 ± 5.9 (9)	268 ± 7.7 (9)	3.40 ± 0.04 (7)	3.62 ± 0.04 (7)	3.68 ± 0.04 (6)	0.22	0.26
N	I	II	40 ± 1.6	189 ± 5.2 (9)	228 ± 12.7 (9)	268 ± 7.2 (7)	3.39 ± 0.02 (7)	3.64 ± 0.02 (7)	3.70 ± 0.05 (6)	0.25	0.32
O	II	I	40 ± 1.7	194 ± 6.6	244 ± 4.8	275 ± 9.0	3.43 ± 0.04 (10)	3.56 ± 0.03 (10)	3.70 ± 0.02 (9)	0.13	0.27
P	II	II	40 ± 2.0	194 ± 7.0	238 ± 7.4	273 ± 11.0	3.38 ± 0.04 (10)	3.55 ± 0.02 (10)	3.68 ± 0.04 (9)	0.17	0.32

[1] Average includes standard error of mean calculated by formula, $\sqrt{\Sigma d^2/n - 1}/\sqrt{n}$.

[2] Where all animals did not survive for 365 days or their x-ray photographs were not available, the differences are calculated only on the rats where complete records were obtained.

and N) and 25 (groups O and P) rats, respectively, were still alive when compared on the basis of dietary history, while 30 (groups M and O) and 25 (groups N and P) survived in the groups when comparisons were based on current diet. These variations are not significant.

Breeding tests were conducted on the 4 groups of rats at approximately 4, 8 and 12 months of age. All progeny

TABLE 4

The fertility and lactation of rats receiving Sherman diet B (groups M and O) or modified Sherman diet (groups N and P)

GROUP	LITTER	FERTILITY			AVERAGE PERIOD AFTER MATING	AVERAGE BODY WT.		YOUNG NOT SURVIVING 3–21 DAYS
		Females bred	Litters	% Fertile		3 days	21 days [1]	
					days	*gm*	*gm*	
M	1	9	9	100	27.3	8.9	42.4 (63)	0
	2	9	7	78	28.6	8.7	37.3 (61)	13
	3	9	1	11	25.0	7.0	50.0 (4)	1
N	1	9	9	100	26.1	9.5	39.2 (21)	1
	2	8	5	62	31.8	8.3	35.5 (35)	0
	3	7	1	14	28.0	..	...	..
O	1	10	9	90	26.0	7.9	33.0 (21)	8
	2	10	2	20	33.0	9.0	42.7 (12)	0
	3	8	0	0	...	..	...	..
P	1	10	9	90	25.3	7.8	33.6 (35)	3
	2	10	6	60	30.0	8.0	32.1 (43)	7
	3	9	2	22	27.0	8.9	43.9 (10)	1

[1] Figures in parentheses indicate the number of rats.

were retained, irrespective of the size of the litter. The data are summarized in table 4.

No striking variations in fertility were noted among the 4 groups at the first breeding. The rats receiving the modified Sherman diet (groups N and P) showed a somewhat better record for fertility in the second and third breedings than those receiving the Sherman diet (groups M and O). Only groups M and P succeeded in raising any progeny from the

third breeding. The mean weights of the rats at weaning were satisfactory with all diets.

An examination of the ash and calcium content of 21-day and 90-day rats on the several diets is reported in table 5.

In the case of the 21-day-old rats, ash made up a somewhat larger proportion of the live weight in the rats from the mothers on the modified Sherman diet (groups N and P) than in those from mothers on the regular Sherman diet (groups M and O). Similarly, the calcium content, based either on the live weight or on the per cent ash, was highest in groups N and P.

On the other hand, in the analyses made on the 90-day-old rats, which were from the same group of litters, a slightly higher percentage of calcium was noted in the rats receiving the original Sherman diet when based on the live weight, but no differences occurred when it was calculated as percentage of ash. A distinct sex difference was observed in the 90-day rats, both in regard to ash and calcium content. The values for ash content were appreciably higher for the female than for the male, irrespective of diet. However, the percentage of calcium in the ash was lower in both cases for the females.

Table 6 records data on the calcium balances of three-month-old rats for the 6-day period before they were sacrificed for the determination of mineral and calcium content recorded in table 5. Analyses of calcium in the food during this period gave values of 0.366 and 0.364% for the Sherman diet B and the modified Sherman diet, respectively.

In all cases, with both male and female rats, positive calcium balances were obtained over a 6-day period. The bulk of the calcium excreted was by way of the intestine and an extremely small proportion was lost in the urine. A somewhat higher positive balance was noted in the males than in the females. To some extent this may be accounted for by differences in body weight and by a higher calcium intake.

Moreover, the positive calcium balance was higher with both male and female groups which received the modified

Sherman diet than with the corresponding groups which were fed the regular Sherman diet B. This variation cannot be ascribed to differences in calcium intake or to variations in body weight among the dietary groups. Although there were too few observations to warrant a statistical analysis, one can at least state that there was no evidence of a poorer

TABLE 5

The ash and calcium content of 21-day-old rats whose mothers had received, previous to weaning, Sherman diet B (I) or modified Sherman diet B (II) and had subsequently been continued on the diets indicated in table during growth, pregnancy and lactation

GROUP	DIET		NUMBER		AVERAGE BODY WEIGHT	ASH [1]		CALCIUM [1]		
	Previous	Current	Males	Females		Weight	% of live weight	Weight	% of live weight	% in
					gm	*gm*		*gm*		
					21-day-old rats					
M	I	I	4	4	37	0.866	2.35 ± 0.04	0.195	0.530 ± 0.007	22.4 ±
N	I	II	4	4	37	0.903	2.44 ± 0.07	0.207	0.564 ± 0.011	23.0 ±
O	II	I	4	4	42	0.906	2.27 ± 0.03	0.206	0.488 ± 0.010	21.5 ±
P	II	II	3	5	35	0.880	2.53 ± 0.05	0.202	0.580 ± 0.012	23.1 ±
					90-day-old rats					
M + O			5		305	8.681	2.97	1.894	0.623	21.7
				5	215	7.717	3.59	1.436	0.669	19.0
N + P			4		297	8.014	2.69	1.649	0.566	21.0
				6	196	6.756	3.45	1.285	0.656	19.1

[1] Average includes standard error of mean calculated by formula, $\sqrt{\Sigma d^2/n - 1}/\sqrt{n}$.

TABLE 6

The calcium intake and excretion of 90-day-old rats which received Sherman diet B (I) or modified Sherman diet B (II) over a 6-day period

DIET	SEX	AVERAGE BODY WEIGHT	AVERAGE FOOD EATEN	CALCIUM				CALCIUM BALANCE
				Eaten	Urine	Feces	Total excretion	
		gm	*gm*	*mg*	*mg*	*mg*	*mg*	
I	Male	305 (5)	60.6	222	9.7	152.2	161.9	+ 60.1 (+ 112.8 to + 13.
	Female	215 (5)	54.0	198	4.3	156.2	160.5	+ 37.5 (+ 68.8 to + 6.
II	Male	297 (4)	66.0	240	3.1	156.8	159.9	+ 85.6 (+ 155.8 to + 48.
	Female	196 (6)	51.0	186	4.3	129.2	133.5	+ 52.0 (+ 88.4 to + 9.

calcium assimilation in rats receiving the diet containing a hydrogenated vegetable fat than in animals on a butterfat-containing ration.

These results are not in agreement with those of Moore ('27), in which better growth and a higher level of bone calcium were noted in puppies when the dietary fat was butter than when the ration contained a vegetable fat of unstated composition. Although the puppies employed were litter mates, the total number of animals used in all the groups was only 8.

It is possible that the differences in results between Moore ('27) and ourselves may be attributed to species difference. Moreover, there is no evidence that the vegetable fat diet used by Moore contained an adequate amount of vitamin D. The butterfat diet might be expected to contain adequate amounts of the antirachitic vitamin.

In the present tests the margarine used contained a minimum of 375 U.S.P. units per pound. Although bioassays for vitamin D were not carried out in our laboratory, reports of periodic bioassays of random samples of the margarine made by an independent laboratory have been supplied to us by the manufacturer. These indicate a minimum level of 375 U.S.P. units per pound.

During the entire course of the multigeneration test, bioassays have been conducted in our laboratory on the vitamin A contained in the same brand of margarine purchased at random on the local market. A minimum of 6 samples a year has been so tested. In all cases the margarines analyzed contained vitamin A in excess of the amount stated on the label; namely 7,500 U.S.P. units previous to about July 1, 1941, then 9,000 U.S.P. units up to approximately July 1, 1945, and finally 15,000 U.S.P. units since the latter date.

The present results give positive evidence that the nutritional status of rats may be maintained at a high level over at least 25 generations when they are fed a diet containing a hydrogenated vegetable margarine as the sole source of dietary fat. The data also indicate that the deposition of

calcium in nursing rats proceeds equally satisfactorily, if not more satisfactorily, when the mothers receive the modified Sherman diet (hydrogenated vegetable margarine) than when they receive Sherman diet B. These results were confirmed by balance studies on three-month-old rats, although no appreciable difference in calcium content existed in the carcasses at that time.

The results of the multigeneration tests over a period of 10 years, and of the studies on calcium deposition and calcium balances, have indicated that a hydrogenated vegetable margarine fat has a nutritive value equal to that of butter when fed with an otherwise adequate diet. The multigeneration tests are being continued with the application of additional biometric measurements.

SUMMARY

The multigeneration experiment with a hydrogenated vegetable margarine fat replacing butterfat in Sherman diet B has now been successfully completed through the 25th generation. Such indices of nutritional value as rate of growth, fertility and lactation have continued at the same high level as was reported earlier for the first 10 generations. Somewhat better growth and lactation were obtained in a "second litter" series started with the 15th generation and continued through the 21st generation than with the main "first litter" series; however, the fertility index was somewhat less satisfactory than with the "first litter" series.

No significant differences in growth, reproduction and lactation were observed between rats maintained on the modified Sherman diet or regular Sherman diet, irrespective of whether the previous nutritional history of their mothers involved a stock diet or the modified Sherman diet over 20 previous generations.

The calcium content of the whole carcasses of 21-day-old rats was higher in animals whose mothers had received a modified Sherman diet than in those where the previous maternal diet had been the usual Sherman regime. Calcium

balances carried out at 90 days showed a somewhat more favorable positive balance for the rats on the Sherman diet modified by the introduction of a hydrogenated vegetable margarine than in those on the original Sherman diet containing butterfat. No similar differences in calcium content ascribable to the diets could be established in the adult rats. It is concluded that a hydrogenated vegetable margarine can supply the fat requirements of the rat over many generations. The employment of such hydrogenated fat results in equally effective calcification as that obtained when animal fat such as butter is present in the diet.

LITERATURE CITED

DEUEL, H. J., JR., L. F. HALLMAN AND E. MOVITT 1945 Studies on the comparative nutritive value of fats. VI. Growth and reproduction over 10 generations on Sherman diet B where butterfat was replaced by a margarine fat. J. Nutrition, *29:* 309.

GREENBERG, S. M., C. E. CALBERT, E. E. SAVAGE AND H. J. DEUEL, JR. 1950 The effect of fat level of the diet on general nutrition. VI. The interrelation of linoleate and linolenate in supplying the essential fatty acid requirement in the rat. Ibid., *41:* 473.

MOORE, C. U. 1927 The value of butter in the diet. Northwest Med., *26:* 29.

SHERMAN, H. C., AND H. L. CAMPBELL 1924 Growth and reproduction upon simplified food supply. IV. Improvement in nutrition resulting from an increased proportion of milk in the diet. J. Biol. Chem., *60:* 5.

———— 1929–1930 Further experiments on the influence of food on longevity. J. Nutrition, *2:* 415.

SHERMAN, H. C., AND H. Y. TRUPP 1949 Further experiments with vitamin A in relation to aging and to length of life. Proc. Nat. Acad. Sci., *35:* 90.

THE NUTRITION OF THE MOUSE

VIII. STUDIES ON PANTOTHENIC ACID, BIOTIN, INOSITOL AND P-AMINOBENZOIC ACID [1]

PAUL F. FENTON,[2] GEORGE R. COWGILL, MARIE A. STONE AND DORIS H. JUSTICE

Yale Nutrition Laboratory, Department of Physiological Chemistry, Yale University, New Haven, Connecticut

(Received for publication June 19, 1950)

We have shown in a previous publication (Fenton and Cowgill, '47) that mice of the A strain possess a higher requirement for riboflavin than do animals of the C_{57} strain. The former show a relatively high susceptibility to spontaneous mammary tumors, while the latter are only slightly susceptible. It seemed advisable to extend this work to other members of the vitamin B complex and to include in the study the C_3H strain of animals, which shows extremely high susceptibility to mammary tumors.

Unna ('40) and Unna et al. ('41) have studied the requirements of the rat for pantothenic acid. They described the deficiency symptoms in some detail and reported that 80 μg of pantothenate per day supported growth as well as did a higher intake of this factor. These findings were confirmed by Henderson et al. ('42). Sandza and Cerecedo ('41) established the need of the mouse for pantothenate and some factor in addition to thiamine, riboflavin and pyridoxine. Norris and Hauschildt ('40) had already reported that the

[1] Supported by a grant from the American Cancer Society upon the recommendation of the Committee on Growth of the National Research Council.

[2] Present address: Department of Biology, Brown University, Providence, Rhode Island.

mouse requires some factor in addition to thiamine, riboflavin, pyridoxine, nicotinic acid and the filtrate factor.

Extensive and careful studies on the pantothenate requirements of C_3H mice have been carried out by Morris and Lippincott ('41–'42) and Lippincott and Morris ('41–'42). These workers concluded that C_3H mice grew as well on a daily intake of 23 to 29 μg as they did on an intake of 200 μg. Since Sandza and Cerecedo ('41) had reported maximum growth of albino mice on daily administration of 30 μg of pantothenate, Morris ('44) concluded that the pantothenate requirements of C_3H and albino mice were identical.

As early as 1940 György and Poling reported that the achromotrichia observed on diets deficient in pantothenate could be largely cured by pantothenate administration, but that complete recovery (the elimination of brownish color and "stippling") occurred only when biotin was also administered. Later Wright and Welch ('43) showed that biotin and folic acid improved the storage of pantothenate in the livers of animals fed diets containing sulfasuxidine. These workers suggested that biotin and folic acid might be concerned in the utilization of pantothenate.

Woolley ('40) postulated the existence of a new dietary factor for the mouse and later showed this to be inositol ('41a). Ansbacher ('41) observed achromotrichia in black rats fed certain purified diets and reported being able to cure this condition by administering *p*-aminobenzoic acid. Martin and Ansbacher ('41a) reported that the feeding of hydroquinone to black mice resulted in achromotrichia curable by the administration of *p*-aminobenzoic acid. In another paper ('41b) they reported the graying of mice on certain rations not containing hydroquinone. Ansbacher's findings with the rat could not be confirmed by Emerson ('41). The controversial reports on both inositol and *p*-aminobenzoic acid suggested the need for further work on these factors, using diets more nearly adequate in the light of recent developments.

TABLE 1

Composition of diets

COMPONENTS	DIET NUMBER														
	131	134	135	136	137	138	174	175	176	177	178	179	180	181	184
Basal mixture															
Casein,[1] gm	30	30	30	30	30	30	30	30	30	30	30	30	30	30	18
Dextrose, gm	45	45	45	45	45	45	..	..	..	..	..	..	..	..	..
Sucrose, gm	..	..	..	..	..	..	53	53	53	53	53	53	53	53	75
Hydrogenated cottonseed oil,[2] gm	15	15	15	15	15	15	..	..	..	..	..	..	..	..	..
Corn oil, gm	..	..	..	..	..	..	10	10	10	10	10	10	10	10	1
Haliver oil, gm	..	..	..	..	..	..	..	..	..	..	..	..	..	..	1
Salt mixture,[3] gm	7	7	7	7	7	7	5	5	5	5	5	5	5	5	5
Ruffex, gm	3	3	3	3	3	3	2	2	2	2	2	2	2	2	..
Supplements															
Cod liver oil concentrate,[4] mg	300	300	300	300	300	300	200	200	200	200	200	200	200	200	..
Linoleic acid, mg	410	410	410	410	410	410	..	..	..	..	..	..	..	..	..
α-Tocopherol, mg	9	9	9	9	9	9	6	6	6	6	6	6	6	6	..
Menadione, mg	..	..	..	..	..	..	1	1	1	1	1	1	1	1	..
Choline, mg	150	150	150	150	150	150	150	150	150	150	150	150	150	150	1
Thiamine, mg	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.2
Riboflavin, mg	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.5
Nicotinic acid, mg	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Pyridoxine, mg	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.2
Folic acid, μg	..	..	..	..	..	..	50	50	50	50	50	50	50	50	..
Biotin, μg	..	..	..	..	..	..	20	20	20	20	20	20	20	20	..
Pantothenic acid, mg	..	0.2	0.4	0.6	0.8	3.0	0.2	0.2	0.2	0.2	6	6	6	6	0.5
PABA, mg	100	100	100	100	100	100	100	..	100	..	100	..	100	..	..
Inositol, mg	100	100	100	100	100	100	100	100	..	..	100	100	..	..	..

[1] Labco, vitamin-free.
[2] Crisco.
[3] Sure's salts no. 2 (Sure, '43).
[4] White's cod liver oil concentrate.

EXPERIMENTAL

Pantothenate — biotin

A total of 117 male mice of three strains (C_{57}, A and C_3H) were used in these studies. All of the young were raised in our own colony under well-standardized conditions and were weaned when 21 days old. At weaning they were placed in individual cages and fed a purified diet containing no added pantothenic acid (diet 131). After one week on this depletion diet the mice were divided into groups which received diets 134 to 138 containing 0.2, 0.4, 0.6, 0.8 and 3.0 mg of pantothenate, respectively, per 100 gm of diet. A few animals of each strain were continued on diet 131. Table 1 shows the composition of these diets.

All mice were weighed daily until their 50th day of life. Some were sacrificed at this time, while others were continued on the experimental diets — some until they were almost 6 months old. Red and white cell counts and hemoglobin determinations were done on tail blood. The pantothenate content of liver and muscle was determined by the method of Skeggs and Wright ('44). In all experiments food and water were supplied ad libitum, but food consumption records were kept for representative animals.

Inositol and p-aminobenzoic acid

A total of 120 weanling mice of the C_{57} and A strains were fed diets 174 to 181 (table 1). Diets 174 to 177 contained an inadequate amount of pantothenic acid, while diets 178 to 181 contained a sufficient amount of this factor. Diets 175 and 179 contained no added *p*-aminobenzoic acid, diets 176 and 180 no added inositol, and diets 177 and 181 neither inositol nor *p*-aminobenzoic acid. The mice were weighed every three days; food and water were supplied ad libitum. A number of mice were maintained on diets 178 to 181 for 30 weeks. Some were sacrificed to permit the weighing of cecal contents and determination of bacterial counts by the method outlined in another paper from this laboratory (Gall

et al., '48). Liver fat was determined by ether extraction of the dried, finely powdered tissue.

Another group of 58 weanlings was fed diet 184, a ration resembling that used by Woolley ('40). When these animals began to lose weight they were divided into two groups, one being fed diet 184 supplemented with 6 mg of pantothenate per 100 gm of diet, and the other diet 184 fortified with 100 mg of inositol per 100 gm.

RESULTS

Pantothenate and biotin

The pertinent data with respect to weight gains, food consumption and blood picture are summarized in table 2. During the 7-day depletion period the average food consumption of mice of all three strains was nearly identical. Despite this fact, the animals of the C_{57} strain gained 4.3 gm, while those of the tumor-susceptible strains gained far less weight. Following the depletion period a few animals of each strain were continued on the pantothenate-free diet. Since there were only a few mice in this completely deficient group, they are not listed in the table. It seems worthwhile to mention that of 4 completely deficient C_{57} mice, one died when 43 days old, another at 45 days, and a third at 47 days. On the other hand, 4 mice of the A and C_3H strains (which gained little weight on the pantothenate-free diet) survived well beyond the 50th day of life.

Mice of the C_{57} strain gained rather appreciable weight even with only 2 μg of pantothenate per gram of diet; maximum growth was obtained when the pantothenate level was raised to 6 μg per gram. On the other hand, mice of the A and C_3H strains grew at a less than maximum rate when the diet contained 8 μg of pantothenate per gram.

The slightly higher than average white blood cell counts found in C_{57} mice on diets low in pantothenate reflect the inclusion of a few animals with considerably elevated counts. C_{57} mice on low pantothenate diets tended to show a higher

TABLE 2

Data obtained with three strains of mice at various dietary levels of pantothenate [1]

Strain	C_{57}					A					C_3H			
Weaning weight, gm	9.3					9.6					10.6			
Depletion period 21–28 days (diet 131)														
Weight gain, gm	4.3					1.4					2.2			
Food consumption, gm	13.1					13.2					13.2			
Supplementation period 28–50 days														
Dietary P. A., μg/gm	2	4	6	8	30	2	4	6	8	30	2	6	8	30
Weight gain, gm	4.8	5.8	8.8	9.2	8.9	0.9	1.7	7.2	7.9	8.9	1.6	6.7	7.9	10.0
Food consumption, gm	35.1	37.3	40.0	41.0	39.7	..	23.3	30.2	35.2	39.3	..	28.0	31.2	38.2
Red cell count, millions [2]	8.4	11.0	10.1	10.3	..	8.6	9.7	8.9	9.4	9.2	8.9	8.7	9.1	9.0
White cell count, thousands [2]	12.5	12.8	7.9	8.4	..	5.4	4.3	6.0	7.9	5.9	5.7	4.8	5.5	5.2
Hemoglobin, gm % [2]	13.3	16.4	16.2	16.7	..	13.3	15.5	14.8	15.0	15.1	14.5	14.7	15.5	15.1

[1] Diets 134, 135, 136, 137, 138 (table 1).
[2] After 50th day.

percentage of lymphocytes than did animals of the same strain receiving the higher levels of the vitamin.

A small number of mice failed to gain weight following the 7-day depletion period despite the fact that their diets then contained adequate quantities of pantothenic acid. As an illustration of this phenomenon we can cite mouse 3595, which weighed 8.9 gm at weaning (21 days). After 7 days on the depletion diet it weighed 7.7 gm. At this time it was fed the diet containing 30 μg of pantothenate per gram. Showing slight weight fluctuations from day to day, this animal still weighed 7.7 gm after one week on the high pantothenate diet. This weight was maintained until the mouse was 43 days old, when it showed a rapid decline in body weight; it died on the 47th day.

The deficiency symptoms observed differed strikingly in the three strains of mice studied. In C_{57} strain mice the onset of achromotrichia occurred with great regularity between the 16th and 22nd days of the experiment. Even the animals which after the initial depletion period received the high pantothenate diet showed, with only one exception, a transient graying of the fur. Hair color returned to normal in these animals in a relatively short time. In mice on the low pantothenate diets the change in hair color was followed later by alopecia, the severity of which was clearly correlated with the amount of pantothenate in the diet. Only one mouse receiving the diet containing 8 μg of pantothenate per gram showed alopecia, while at the 30-μg level no hair loss was observed in any animal. The pattern of color change and alopecia in the C_{57} strain was very uniform from one animal to another. Graying was first observed on the flanks and was then seen to spread out over the dorsal body area. The head and a small area around the base of the tail were the last areas to be affected. In curative tests these two areas were usually the last to recover their normal pigmentation. Alopecia, when it occurred, was seen first on the flanks and then, if the diet was very low in pantothenate, would spread over the dorsal area. Only rarely did it extend over the

ventral surface unless the experiment was carried beyond the 50th day of life. Similarly, dermatosis and partial paralysis of the hind legs were observed only on prolonged feeding of the low-pantothenate diets. In some mice of the C_3H strain a change in hair color could be noted on careful observation. More frequently the change involved only a loss of luster. Alopecia was noted in about one-half of the C_3H mice receiving the low pantothenate diets. It was never seen in this strain when the higher pantothenate levels were fed (8 or 30 μg per gram). In the A strain very little alopecia could be observed prior to the 50th day of life. Priapism was occasionally noted in mice of the C_3H and A strains maintained on diets low in pantothenate.

Analysis of liver and muscle tissue for pantothenic acid showed that the level of this vitamin in the tissues of C_{57} mice on low pantothenate diets was lower than in mice receiving higher levels. In A and C_3H strain mice the liver pantothenate levels did not differ greatly on the various diets. Relatively few assays were carried out on the muscle tissues of these two strains.

It was noted particularly in mice of the C_{57} strain, which were continued on the experiment past the 50th day of life, that after the period of most rapid growth had passed spontaneous restoration of hair and hair color could occur. It was thought that the administration of biotin might aid in the recovery process. To test this possibility, 5 μg of biotin were administered subcutaneously on alternate days for 38 days. While the interpretation of the response must necessarily be highly subjective, it was clear to several observers that the biotin-treated animals recovered more rapidly than those animals which were showing spontaneous recoveries.

Mice which were continued on low pantothenate diets beyond the 50th day of life showed the following deficiency symptoms: achromotrichia (in C_{57} strain mice), alopecia, dermatosis, and paralysis of the hind legs (dragging gait). In none of the animals did we observe porphyrin-containing

exudates or grossly abnormal adrenals. Some adrenals were sectioned with the freezing microtome but no histologic abnormalities could be found.

Inositol and p-aminobenzoic acid

Table 3 shows the weight gains of the several experimental groups. All those receiving the low pantothenate diets grew more slowly than those receiving adequate amounts of this factor. On the low pantothenate diets the C_{57} mice grew appreciably better than did the animals of the A strain.

TABLE 3

Average body weights on diets with and without inositol or p-aminobenzoic acid

DIET	INOSITOL	PABA	STRAIN C_{57}, AT AGE			STRAIN A, AT AGE		
			21 days	33 days	42 days	21 days	33 days	42 days
			gm	*gm*	*gm*	*gm*	*gm*	*gm*
174	+	+	10.7	16.6	17.7	11.8	14.1	14.3
175	+	—	11.1	18.3	19.2	10.2	13.5	13.8
176	—	+	10.9	17.6	18.4	10.1	12.2	11.8
177	—	—	11.0	17.4	17.3	11.5	13.4	13.3
178	+	+	11.5	20.7	23.1	11.7	20.4	23.8
179	+	—	11.8	22.3	23.4	11.7	20.9	24.4
180	—	+	10.8	21.2	22.8	12.0	19.9	23.1
181	—	—	11.3	21.1	23.0	12.0	21.9	24.4

This finding corroborates those of the first part of this paper. The presence or absence of inositol or *p*-aminobenzoic acid did not appear to influence the rate of growth. No outward symptoms were observed that could be attributed to a deficiency in these factors. Blood counts on a relatively few animals showed no alteration in the blood picture due to the absence of inositol or *p*-aminobenzoic acid. Liver fat tended to be slightly higher in animals receiving both factors than in animals deprived of one or both.

The weight of the cecal contents was higher in mice of the C_{57} strain than in those of the A strain, while the number of organisms per gram of cecal content was the same in

the two strains. The presence or absence of inositol or *p*-aminobenzoic acid had no effect. The weight of the cecal content and the number of organisms per gram of content were appreciably higher in animals fed a commercial stock ration than in mice fed the purified diets.

Mice placed on diet 184 (low in pantothenate and in other factors) grew slowly at first and then lost weight. When the diet was supplemented with additional pantothenate, growth was resumed. The addition of inositol to the diet temporarily stimulated growth in some animals but not in all.

DISCUSSION

Pantothenate and biotin

Under the conditions of these experiments mice of the C_{57} strain grew at the maximum rate when the diet contained 6 μg of pantothenate per gram. Neither C_3H nor A strain mice grew at the maximum rate even when the diet contained 8 μg of pantothenate per gram. It is interesting to note that these results tend to confirm Morris' conclusions ('44) that mice of the A and C_3H strains possess approximately the same requirements for pantothenic acid. The lower pantothenate requirement of the C_{57} strain distinguishes this strain from the other two, a situation we have found to exist also with respect to the riboflavin requirement.

It is of interest to note that during the one-week depletion period the food consumption of the three strains was identical, yet the C_{57} mice gained considerably more weight than the others. This ability of the C_{57} strain to gain more weight per unit weight of food consumed has been observed in connection with other diets and is being investigated more fully. The fact that rapidly growing animals receiving diets low in pantothenate show more severe deficiency symptoms than more slowly growing animals may perhaps be explained on the basis of greater metabolic utilization and destruction of pantothenate during rapid growth. It has been shown in these experiments that mice receiving low pantothenate diets

may spontaneously recover from the obvious signs of deficiency when the growth rate declines. Similar observations have been made by Emerson and Evans ('41) and by Unna and Richards ('42). Our observation that subsequent to the week's depletion period even mice fed the diet high in pantothenate may show transitory graying reveals not only the declining pantothenate requirement with age but emphasizes the ease with which pantothenate deficiency may be induced. The more rapid recovery from pantothenate deficiency when biotin was administered in addition to pantothenate confirms the earlier finding of György and Poling ('40).

Inositol and p-aminobenzoic acid

Diets presumably complete in all factors except inositol and *p*-aminobenzoic acid did not permit the demonstration of a deficiency in either of these factors. Only with the inadequate diet 184 could a dubious inositol response be obtained. The pattern of alopecia seen in our pantothenate-deficient mice was similar to that described by Woolley ('40). This pattern was seen in the experiments reported in the first part of this paper on diets low in pantothenate but containing presumably adequate amounts of inositol. It seems impossible, therefore, to distinguish between pantothenate and inositol deficiencies on the basis of the alopecia pattern. If Woolley's interpretation ('41b, '42) of the effect of pantothenate on inositol deficiency can be accepted, one would not expect to see inositol deficiency on diet 180, which is relatively rich in pantothenate.

In connection with the studies on the cecal flora it is of interest to point out that the observations confirm those reported earlier from this laboratory (Gall et al., '47). The presence or absence of inositol or *p*-aminobenzoic acid appeared to have no influence on the number of organisms present. No effort was made, however, to investigate the bacterial synthesis of inositol, *p*-aminobenzoic acid or pantothenate under the conditions employed in these experiments.

While it is apparent that inositol and *p*-aminobenzoic acid may be safely omitted from diets which are adequate in all other respects, it is by no means certain that this omission can be made under all nutritional conditions.

SUMMARY AND CONCLUSIONS

1. Mice of the C_{57} strain grew at the maximum rate under the conditions employed when the diet contained 6 μg of pantothenate per gram. A and C_3H mice did not grow at quite the maximum rate when the diet contained 8 μg of pantothenate per gram.

2. Biotin was observed to aid in the restoration of hair and hair color.

3. No deficiency of inositol or *p*-aminobenzoic acid could be demonstrated when the diet contained adequate amounts of pantothenic acid and was presumably complete in all other respects.

ACKNOWLEDGMENTS

We wish to thank Dr. R. J. Floody of Hoffmann-La Roche, Inc. for supplying the biotin and Dr. T. H. Jukes of the Lederle Laboratories for the folic acid used in these studies.

LITERATURE CITED

ANSBACHER, S. 1941 *p*-Aminobenzoic acid, a vitamin. Science, *93:* 164.

EMERSON, G. A. 1941 Failure to cure or prevent graying of rats with *p*-aminobenzoic acid. Proc. Soc. Exp. Biol. Med., *47:* 448.

EMERSON, G. A., AND H. M. EVANS 1941 Growth and graying of rats with total "filtrate factor" and with pantothenic acid. Ibid., *46:* 655.

FENTON, P. F., AND G. R. COWGILL 1947 The nutrition of the mouse. I. A difference in the riboflavin requirements of two highly inbred strains. J. Nutrition, *34:* 273.

GALL, L. S., P. F. FENTON AND G. R. COWGILL 1948 The nutrition of the mouse. II. Effect of diet on the bacterial flora of the small intestine and cecum. Ibid., *35:* 13.

———— 1947 Nutrition of the mouse. IV. Comparison of bacterial population of two highly inbred strains. Proc. Soc. Exp. Biol. Med., *66:* 414.

GYÖRGY, P., AND C. E. POLING 1940 Further experiments on nutritional achromotrichia in rats and mice. Ibid., *45:* 773.

HENDERSON, L. M., J. M. MCINTIRE, H. A. WAISMAN AND C. A. ELVEHJEM 1942 Pantothenic acid in the nutrition of the rat. J. Nutrition, *23:* 47.

LIPPINCOTT, S. W., AND H. P. MORRIS 1941–42 Morphologic changes associated with pantothenic acid deficiency in the mouse. J. Nat. Cancer Inst., *2:* 39.

MARTIN, G. J., AND S. ANSBACHER 1941a Confirmatory evidence of the chromotrichial activity of *p*-aminobenzoic acid. J. Biol. Chem., *138:* 441.

———— 1941b Rôle of para-aminobenzoic acid in vitamin B-complex studies with mice. Proc. Soc. Exp. Biol. Med., *48:* 118.

MORRIS, H. P. 1944 Review of the nutritive requirements of normal mice for growth, maintenance, reproduction, and lactation. J. Nat. Cancer Inst., *5:* 115.

MORRIS, H. P., AND S. W. LIPPINCOTT 1941–42 The effect of pantothenic acid on growth and maintenance of life in mice of the C_3H strain. Ibid., *2:* 29.

NORRIS, E. R., AND J. HAUSCHILDT 1940 A new factor of the vitamin B complex required by the albino mouse. Science, *92:* 316.

SANDZA, J. G., AND L. R. CERECEDO 1941 Requirement of the mouse for pantothenic acid and for a new factor of the vitamin B complex. J. Nutrition, *21:* 609.

SKEGGS, H. R., AND L. D. WRIGHT 1944 The use of *Lactobacillus arabinosus* in the microbiological determination of pantothenic acid. J. Biol. Chem., *156:* 21.

SURE, B. 1943 Dietary requirements for fertility and lactation. XXXI. Further studies on the role of *p*-aminobenzoic acid and inositol in lactation and growth of the albino rat. J. Nutrition, *26:* 275.

UNNA, K. 1940 Pantothenic acid requirement of the rat. Ibid., *20:* 565.

UNNA, K., AND G. V. RICHARDS 1942 Relationship between pantothenic acid requirement and age in the rat. Ibid., *23:* 545.

UNNA, K., G. V. RICHARDS AND W. L. SAMPSON 1941 Studies on nutritional achromotrichia in rats. Ibid., *22:* 553.

WOOLLEY, D. W. 1940 A new dietary essential for the mouse. J. Biol. Chem., *136:* 113.

———— 1941a Identification of the mouse antialopecia factor. Ibid., *139:* 29.

———— 1941b Relationship of pantothenic acid and inositol to alopecia in mice. Proc. Soc. Exp. Biol. Med., *46:* 565.

———— 1942 Synthesis of inositol in mice. J. Exp. Med., *75:* 277.

WRIGHT, L. D., AND A. D. WELCH 1943 The role of "folic acid" and biotin in the utilization of pantothenic acid by the rat. Science, *97:* 426.

COMPARATIVE EFFECTS OF B VITAMINS AND LIVER ON DINITROPHENOL TOXICITY IN THE RAT [1]

BENJAMIN H. ERSHOFF

Emory W. Thurston Laboratories, Los Angeles, and the Department of Biochemistry and Nutrition, University of Southern California, Los Angeles

(Received for publication May 31, 1950)

It is becoming increasingly apparent that the effects obtained in an experimental animal following administration of drugs or related products are dependent on the nutritional state and the diets employed. In acute deficiencies of essential nutrients it is readily recognized that resulting abnormalities in cellular metabolism may profoundly affect response to certain drugs. What is less well recognized, however, is that drugs themselves may precipitate a deficiency state. Thyroid administration, for example, may induce a deficiency of thiamine, pyridoxine and pantothenic acid (Drill and Overman, '42), while dicumarol, salicylates and other drugs may precipitate a deficiency of vitamin K (Shapiro et al., '43; Shapiro, '44; Collins and Hoffmann, '43).

In addition to the known nutrients, requirements for various unknown factors may also be increased following the administration of certain drugs. These "minor vitamins" are apparently dispensable under normal conditions, or requirements for them are so small they may readily be met by amounts present in the diet or through the synthetic activity of the intestinal flora or the animal's own tissues. Following drug administration, however, requirements for these substances may be increased to the extent that deficiencies occur,

[1] Communication no. 261 from the Department of Biochemistry and Nutrition, University of Southern California.

manifested by retarded growth or tissue pathology and preventable by the administration in appropriate amounts of the missing nutrient (Ershoff, '48a). Whole liver is a potent source of such unknown nutrients. Thus whole liver or fractions thereof has been shown to counteract the deleterious effects of massive doses of strychnine (Battelli, '40), sulfanilamide (Chamelin and Funk, '43), promin (Higgins, '44; Ershoff and McWilliams, '49) and atabrine (Ershoff, '48b, '50). Similar results have been observed following the administration of toxic doses of diethyl-stilbestrol (Chamelin and Funk, '43), alpha-estradiol (Ershoff and McWilliams, '48a), desiccated thyroid (Ershoff, '47a, b; Betheil et al., '47), and thyroxin, thyroglobulin and iodinated casein (Ershoff and McWilliams, '48b). In the present paper data are presented on the comparative effects of liver and the known B vitamins on dinitrophenol toxicity in the immature rat.

PROCEDURE AND RESULTS

The basal ration employed in the present experiment consisted of sucrose, 73.4%; casein,[2] 22.0%; salt mixture,[3] 4.5%; and dinitrophenol,[4] 0.1%. To each kilogram of the above were added the following synthetic vitamins: thiamine hydrochloride, 72 mg; riboflavin, 9 mg; pyridoxine hydrochloride, 15 mg; calcium pantothenate, 67.2 mg; nicotinic acid, 60 mg; 2-methylnaphthoquinone, 5 mg; and choline chloride, 1.2 gm. Each rat also received three times weekly the following supplement: cottonseed oil,[5] 500 mg; alpha-tocopherol acetate, 1.5 mg; and a vitamin A–D concentrate containing 50 U. S. P. units of vitamin A and 5 U. S. P. units of vitamin D.[6] In addition to the basal ration the following diets were also employed: (1) basal ration plus the following vitamin supplements per

[2] Vitamin-test casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[3] Hubbel, Mendel and Wakeman salt mixture, General Biochemicals, Inc., Chagrin Falls, Ohio.

[4] Material used was 2,4-dinitrophenol, Eastman Kodak Co., Rochester, N. Y.

[5] Wesson.

[6] Nopco fish oil concentrate, assaying 800,000 U. S. P. units of vitamin A and 80,000 U. S. P. units of vitamin D per gram.

kilogram of diet: thiamine hydrochloride, 20 mg; riboflavin, 20 mg; pyridoxine hydrochloride, 20 mg; calcium pantothenate, 60 mg; nicotinic acid, 60 mg; biotin, 4 mg; folic acid, 10 mg; *p*-aminobenzoic acid, 400 mg; inositol, 800 mg; 2-methyl-naphthoquinone, 10 mg; and vitamin B_{12},[7] 30 μg; (2) basal ration plus 10% whole liver powder;[8] (3) basal ration plus 10% extracted liver residue;[9] and (4) basal ration plus 4% liver concentrate powder 1–20.[10] The vitamin supplements and liver fractions were added in place of an equal amount of sucrose. In addition to the above diets a control ration was also tested, which consisted of the basal ration with dinitrophenol omitted and replaced by an equal amount of sucrose.

Sixty male rats of the Long-Evans strain were selected for the present experiment at 21 to 23 days of age and an average weight of 46.3 gm. Animals were kept in metal cages with raised screen bottoms to prevent access to feces and were fed the above diets ad libitum. Feeding was continued for 28 days. The results are summarized in table 1.

Findings indicate that the effects of dinitrophenol feeding on the immature rat are dependent on the diet employed. Body and testicular weights were significantly less in animals fed the basal ration than in those fed a similar diet with dinitrophenol omitted. Whole liver powder, when added to the basal ration, resulted in a significant increase in body and testicular weight. Supplements of the known B vitamins, however, had little, if any, effect. Some increase in body and gonadal weight was observed following the administration of liver concentrate 1–20 or water-insoluble liver residue. Gain in body and testicular weight on these supplements, however, was not as marked as that obtained with whole liver powder. No significant difference in ventricular weight, when

[7] Cobione (crystalline vitamin B_{12}), Merck and Co., Rahway, N. J.

[8] Whole dried liver powder, Armour and Co., Chicago, Ill.

[9] Extracted liver residue, Wilson Laboratories, Chicago, Ill. This consists of the coagulated, water-insoluble material remaining after the removal of the extractable water-soluble substances.

[10] Liver concentrate powder 1–20, Wilson Laboratories, Chicago, Ill. This consists of the water-extractable material of raw liver.

expressed in terms of milligrams per 100 gm body weight, was observed among the animals fed the various diets. This finding is in accord with the report of Terada and Tainter ('35) that doses of dinitrophenol similar to those employed in the present experiment cause little or no stimulation of

TABLE 1

Comparative effects of B vitamins and liver on body, testicular and ventricular weights of immature rats fed massive doses of dinitrophenol (10 animals per group)

SUPPLEMENTS FED WITH BASAL RATION	DINITROPHENOL IN RATION	INITIAL BODY WT.	AVE. GAIN IN BODY WT. AFTER 28 DAYS OF FEEDING [1]	AVE. TESTICULAR WT.[1]	AVE. VENTRICULAR WT.
	%	*gm*	*gm*	*gm*	*mg/100 gm body wt.*
None	0.1	47.0	40.9 ± 3.2	0.82 ± 0.06	360
B vitamins	0.1	46.3	57.0 ± 4.1	0.87 ± 0.06	377
Liver concentrate powder 1–20	0.1	46.1	65.2 ± 3.5	1.17 ± 0.10	366
Extracted liver residue	0.1	46.0	70.9 ± 3.3	1.58 ± 0.11	372
Whole liver powder	0.1	46.3	95.4 ± 4.7	1.91 ± 0.15	370
None	0.0	45.8	99.3 ± 3.9	1.98 ± 0.13	379

[1] Including standard error of the mean calculated as follows: $\sqrt{\frac{\Sigma d^2}{n}} / \sqrt{n}$, where "d" is the deviation from the mean and "n" is the number of observations.

metabolism in the immature rat.[11] All rats survived the experimental period of 28 days. With the exception of body and gonadal weight, no significant differences were observed grossly among animals fed the various diets.

[11] Food intake data similarly suggest that the metabolic rate was not increased. The average caloric intake during the first 10 days of rats fed diets containing dinitrophenol ranged from 5% to 30% below that of animals fed the basal ration with dinitrophenol omitted. In contrast to the above, food consumption is generally increased under conditions of accelerated metabolism. Food consumption was not determined after the 10th day of feeding.

DISCUSSION

Considerable data are available indicating that many of the effects of drug intoxication are due not to excessive doses of the drug per se but rather to nutritional deficiencies which are induced by its prolonged administration. These nutrients may be employed for purposes of detoxication, or they may form parts of enzyme systems concerned in the detoxication of a particular drug. In some cases nutritional deficiencies may result from impaired absorption or utilization or increased excretion or destruction resulting from drug therapy. The net result, however, is that nutritional requirements are increased beyond the usual or average range. Furthermore, these effects may occur in animals that are normal at the start of the experiment and that are fed diets which are qualitatively and quantitatively adequate in the absence of the drugs employed (Ershoff, '48a).

The findings of the present study suggest that the retardation in growth and inhibition of testicular development of immature rats fed massive doses of dinitrophenol were due, under the conditions of the present experiment, to a nutritional deficiency and were not primary effects of drug intoxication. This is indicated by the fact that these effects were completely counteracted by the administration of dessiccated whole liver. No data are available, however, to indicate what factor or factors in whole liver were responsible for the protective effect. Neither the water-soluble fraction of liver (liver concentrate powder 1–20) nor its water-insoluble residue (extracted liver residue) were as effective as whole liver in counteracting the symptoms of dinitrophenol toxicity. In addition, supplements of all the known B vitamins had little, if any, effect. The vitamin B_{12} content of the whole liver ration, however, was considerably greater than the B_{12} content of the diet containing the synthetic vitamin supplements. Each gram of desiccated whole liver contained (according to the manufacturer) approximately 1.1 μg of "vitamin B_{12}-like activity." The vitamin B_{12} content of the whole liver ration (which contained 10% desiccated whole liver) was, accord-

ingly, approximately 110 μg per kilogram of diet, in contrast to the 30 μg per kilogram in the ration containing the synthetic B vitamin supplements. It is possible, therefore, that the protective effects of the whole liver ration may have been due, at least in part, to its higher vitamin B_{12} content. However, both body and gonadal weights of the rats fed the liver concentrate 1–20 diet were significantly smaller than those of animals fed the whole liver ration, although the vitamin B_{12} content of the liver concentrate diet was equal to, and possibly greater than, that of the whole liver ration.[12] Furthermore, both the body and gonadal weights of the rats fed the liver residue diet were greater than those of animals fed the liver concentrate 1–20 ration, although the B_{12} content of the latter was three to 5 times greater than that of the former. It would appear, therefore, that in addition to vitamin B_{12}, whole liver contains one or more nutrients, presumably distinct from any of the known B vitamins, whose requirement is increased in immature rats fed massive doses of dinitrophenol. Further work is indicated to determine to what extent protein or other known nutrients in liver may have contributed to the observed results.

SUMMARY

A marked retardation in body and testicular weights was observed in immature rats fed a purified ration containing 0.1% dinitrophenol. These effects were completely counteracted by the administration of desiccated whole liver when fed at a level of 10% of the diet. Supplements of all the known B vitamins had little, if any, effect.

[12] According to data supplied by the manufacturer, the "vitamin B_{12}-like activity" of liver concentrate powder 1–20 ranged from 3.0 to 5.0 μg/gm, and that of extracted liver residue from 0.2 to 0.4 μg/gm. The vitamin B_{12} content of the ration containing 4% liver concentrate powder 1–20 was therefore approximately 120 to 200 μg per kilogram of diet, while the B_{12} content of the ration containing 10% extracted liver residue was approximately 20 to 40 μg per kilogram of diet.

LITERATURE CITED

BATTELLI, G. 1940 Detoxicating action of liver extracts in experimental strychnine poisioning. Boll. Soc. ital. biol. sper., *15:* 687; through Chem. Abstr., 1946, *40:* 5833.

BETHEIL, J. J., V. D. WIEBELHAUS AND H. A. LARDY 1947 Studies of thyroid toxicity. I. A nutritional factor which alleviates the toxicity of ingested thyroid substance. J. Nutrition, *34:* 431.

CHAMELIN, I. M., AND C. FUNK 1943 The action of liver extracts in counteracting the toxic effects of diethyl-stilbestrol and sulfanilamide. Arch. Biochem., *2:* 9.

COLLINS, E. N., AND A. D. HOFFMANN 1943 Hemoptysis and hematuria in sprue. Cleveland Clin. Quart., *10:* 105.

DRILL, V. A., AND R. OVERMAN 1942 Increased requirements of pantothenic acid and vitamin B_6 during experimental hyperthyroidism. Am. J. Physiol., *135:* 474.

ERSHOFF, B. H. 1947a Effects of liver feeding on growth and ovarian development in the hyperthyroid rat. Proc. Soc. Exp. Biol. Med., *64:* 500.

———— 1947b Comparative effects of liver and yeast on growth and length of survival of the immature thyroid-fed rat. Arch. Biochem., *15:* 365.

———— 1948a Conditioning factors in nutritional disease. Physiol. Rev., *28:* 107.

———— 1948b The effects of B vitamins, liver and yeast on atabrine toxicity in the rat. J. Nutrition, *35:* 269.

———— 1950 Growth-promoting activity of vitamin B_{12} in mice fed massive doses of atabrine. Arch. Biochem., *26:* 50.

ERSHOFF, B. H., AND H. B. MCWILLIAMS 1948a Effects of B vitamin, yeast and liver on ovaries of immature rats fed massive doses of alpha-estradiol. Proc. Soc. Exp. Biol. Med., *67:* 323.

———— 1948b Effects of the antithyrotoxic factor of liver on growth and survival of immature rats fed massive doses of thyroid-active materials. Science, *108:* 632.

———— 1949 The effects of B vitamins, liver, and yeast on promin toxicity in the rat. J. Am. Pharm. Assn., *38:* 274.

HIGGINS, G. M. 1944 The influence of purified diets on the toxicity of promin. Am. J. Clin. Path., *14:* 278.

SHAPIRO, S. 1944 Studies on prothrombin. VI. The effect of synthetic vitamin K on the hypoprothrombinemia induced by salicylate in man. J. Am. Med. Assn., *125:* 546.

SHAPIRO, S., M. H. REDISH AND H. A. CAMPBELL 1943 Prothrombin studies. III. Effect of vitamin K upon hypoprothrombinemia induced by dicumarol in man. Proc. Soc. Exp. Biol. Med., *52:* 12.

TERADA, B., AND M. L. TAINTER 1935 Metabolic response of white rats to continued administration of dinitrophenol. J. Pharm. Exp. Ther., *54:* 454.

THE PROTECTIVE EFFECT OF HIGH FAT DIETS ON IMMATURE RATS FED THYROID [1,2]

SAMUEL M. GREENBERG AND HARRY J. DEUEL, JR.
Department of Biochemistry and Nutrition, University of Southern California, Los Angeles

(Received for publication June 5, 1950)

Stress factors in the environment or in the metabolism of experimental animals have been used in recent years to accelerate the appearance of deficiency symptoms that might otherwise be delayed or never appear as obvious manifestations.

In this laboratory it was demonstrated earlier by Ershoff and Deuel ('45) that deficiencies in diet could be accentuated by the administration of the growth hormone. In vitamin A-depleted rats the administration of this hormone markedly decreases survival time and hastens the appearance of acute vitamin A deficiency symptoms.

On the other hand, Deuel and co-workers ('50) found that symptoms of essential fatty acid deficiency were not augmented by the growth hormone. However, the potency of the growth hormone was established by demonstrating an increased rate of growth when linoleic acid was present. Decker and Mead ('49) report that both chronic and acute essential fatty acid deficiency markedly increased the mortality rate of mice exposed to x-ray radiation. More recently these workers (Decker and Mead, '50) have found that minor injury, such as the withdrawal of a small amount of blood

[1] Supported by a grant from the Best Foods, Inc.
[2] Contribution no. 264 from the Department of Biochemistry and Nutrition, University of Southern California.

from essential fatty acid-deficient mice, will cause the symptoms of such deficiency to appear rapidly.

It is well known that the feeding of large doses of thyroid increases the requirement for some of the B vitamins (Ershoff, '49b). Yeast has been shown to prolong the survival of rats fed massive doses of thyroid (Ershoff and Hershberg, '45), while desiccated and defatted whole liver have been found not only to increase survival (Ershoff, '47a) but also

TABLE 1

The composition of the diets employed in the experiments

DIETARY COMPONENTS	DIET		
	69a	69b	70a
Casein, vitamin-test [1]	20	20	27.8
Sucrose	71.67	71.34	31.74
Cottonseed oil [2]	0	0	30.0
Cellulose [3]	4	4	5.0
Salt mixture [4]	4	4	5.0
Water-soluble vitamin mixture [5]	0.33	0.66	0.46

[1] General Biochemicals, Inc.

[2] Wesson oil.

[3] Cellu flour obtained from the Chicago Dietetic Supply House.

[4] Osborne-Mendel salt mixture (J. Biol. Chem., '17, *32:* 309).

[5] The water-soluble vitamin mixture had the following percentage composition: choline and inositol, each 36.70; para-aminobenzoic acid, 18.35; thiamine chloride hydrochloride, 2.20; calcium pantothenate, 2.06; nicotinic acid, 1.83; riboflavin and pyridoxine hydrochloride, each 0.83; folic acid, 0.31; 2-methylnaphthoquinone, 0.15; and biotin, 0.02. The biotin was kindly furnished by Hoffmann-La Roche and the folic acid by Lederle Laboratories.

to counteract completely growth retardation in immature rats (Ershoff, '47b). In rations containing soybean meal as a source of protein, Emerson ('49) found that vitamin B_{12} was able to prevent the depressing effect on growth of massive doses of thyroid. However, when casein was the dietary protein and sucrose the carbohydrate component, as in the present tests, growth retardation caused by a large intake of thyroid could be only partly alleviated by the administra-

tion of vitamin B_{12} (Ershoff, '49a). Soybean oil and vitamin B_{12} were found to be equally effective for counteracting a deficiency in vitamin B_{12} induced by thyroid feeding in animals fed a diet containing soybean meal as the principal source of protein.

TABLE 2

Mean weights and percentage mortality of rats on following diets: I, fat-low; II, fat-low containing 0.05% desiccated thyroid powder; III, fat-low containing 0.05% desiccated thyroid powder and with twice the water-soluble vitamin content; IV, 30% cottonseed oil diet; V, 30% cottonseed oil diet containing 0.05% desiccated thyroid (6 animals in each group)

DIET	BEGINNING WEIGHT	WEIGHT AT END OF FOLLOWING WEEKS				MORTALITY
		3	6[1]	9	11	
	gm	gm	gm	gm	gm	%
			Male rats			
I	41.1	112.8	173.3 ± 4.0	190.5	203.3	0
II	43.7	104.8	138.7 ± 2.4	135.2	141.7	50
				(5)	(3)	
III	44.0	102.2	114.0 ± 3.1	130.0	134.0	83
			(4)	(3)	(1)	
IV	41.8	134.5	207.0 ± 14.9	251.5	271.7	0
V	43.8	138.0	216.3 ± 7.5	260.8	280.2	0
			Female rats			
I	46.3	106.7	134.7 ± 7.4	153.7	161.0	0
II	45.3	103.2	135.2 ± 4.1	126.8	121.0	50
			(5)	(5)	(3)	
III	43.8	89.5	117.5 ± 10.5	127.5	135.0	83
			(4)	(2)	(1)	
IV	44.7	122.0	166.5 ± 3.9	188.8	200.7	0
V	43.8	124.7	175.8 ± 7.4	194.8	208.3	0

[1] Includes the standard error of the mean calculated by the formula $\sqrt{\Sigma d^2/n-1}/\sqrt{n}$, where d = deviation from the mean and n = the number of observations.

The present experiments were designed to develop a method for the acceleration of fat deficiency by the use of thyroid as a stress factor.

EXPERIMENTAL

Weanling rats of the U.S.C. strain were used. Six male and 6 female animals were assigned to each of 5 groups. The diets fed are given in table 1. Vitamins A and D and

α-tocopherol were supplied in alcohol solution, the daily dosage being: vitamin A — 50 U.S.P. units; vitamin D — 5 U.S.P. units; and α-tocopherol — 0.5 mg. The vitamin supplement was administered twice weekly. The fat-low basal diet (69a) and the high-fat basal diet (70a) were employed in groups I and IV, respectively; diet 69b used for group III is similar to diet 69a except that the water-soluble vitamin content is doubled. Groups II, III and V received diets to which was added 0.05% of desiccated thyroid powder.[3] The animals were housed in individual raised screen cages and were weighed weekly. Food and water were given ad libitum. The weights of the animals and the mortality in each group are given for representative periods in table 2. The standard error of the mean weight was determined at the 6th week instead of at a later period since a large number of the animals in each group were still alive at that stage of the experiment.

DISCUSSION

The data indicate that immature rats on a fat-low diet are particularly susceptible to thyroid feeding, as is evidenced by a greater retardation in growth and a decreased length of survival. On the other hand, when generous amounts of cottonseed oil are added to the diet, no deleterious effects of desiccated thyroid on growth or survival time are noted. Thus, the gain in body weight of male and female rats on a high-fat diet was equal whether or not thyroid was added; in the tests with the low-fat diet the body weights in the thyroid-fed groups were lower in practically all cases. Moreover, a marked mortality obtained in the thyroid-fed animals (group II) which appeared to be accentuated when the amounts of the water-soluble vitamins were increased (group III). The latter phenomenon may indicate that when the extra requirement for the B vitamins caused by the thyroid is met, an opportunity for a further accentuation of the stress occurs.

[3] Armour, U.S.P. thyroid powder.

The present experiments are of interest because of the close relationship between the effects of fat in preventing the sensitivity of rats on a low-fat diet to thyroid feeding and the effects of soybean oil in alleviating thyroid toxicity in experiments in which diets containing low-fat soybean flour were used (Ershoff, '49b). However, in the present tests, where casein was used, the depressing effects of the thyroid preparation were entirely suppressed by cottonseed oil, in contradistinction to the partial effect of soybean oil in the tests where casein was the dietary protein. In the experiments of Ershoff, the animals on the low-fat diet received 1,500 mg of cottonseed oil weekly, while in our tests no dietary fat was given in the cases of groups I, II, and III. Although our water-soluble vitamin mixture did not contain vitamin B_{12}, Ershoff ('49b) failed to note any effect of this vitamin in a diet similar to the present one.

It is of considerable importance that the harmful effects of thyroid powder are noted on fat-low diets with such small doses. In the present tests the thyroid preparation was given at a level of only 0.05%, compared with the levels of 0.25% and 0.50% employed by Ershoff ('49b). This may be of clinical significance with respect to relatively normal individuals. Experiments are in progress to determine the level of fat necessary to overcome the deleterious effect of thyroid, as well as to ascertain whether qualitative differences exist among various fats in their therapeutic action.

SUMMARY

The retardation in growth of immature rats fed a diet deficient in fat was accentuated by the administration of relatively low dosages of desiccated thyroid. Thyroid feeding also significantly reduced the average length of survival of rats fed a diet deficient in fat. These deleterious effects did not occur when a similar diet containing 30% of cottonseed oil was given.

LITERATURE CITED

DECKER, A. B., AND J. F. MEAD 1949 The relationship between essential fatty acid deficiency and radiation damage in the mouse. U. S. Atomic Energy Project Report, U.C.L.A., no. 52.

———— 1950 Chronic essential fatty acid deficiency. I. Its effect on fertility and wound healing. U. S. Atomic Energy Project Report, U.C.L.A., no. 65.

DEUEL, H. J., JR., S. M. GREENBERG, C. E. CALBERT, E. E. SAVAGE AND T. FUKUI 1950 The effect of fat level of the diet on general nutrition. II. The relationship of linoleic acid requirement to optimum fat level in the diet. J. Nutrition, *40:* 351.

EMERSON, G. A. 1949 Growth promoting activity of vitamin B_{12} in rats receiving thyroid substance. Proc. Soc. Exp. Biol. Med., *70:* 392.

ERSHOFF, B. H. 1947a Comparative effects of liver and yeast on growth and length of survival of the immature thyroid-fed rat. Arch. Biochem., *15:* 365.

———— 1947b Effects of liver feeding on growth and ovarian development in the hyperthyroid rat. Proc. Soc. Exp. Biol. Med., *64:* 500.

———— 1949a An antithyrotoxic factor for the rat not identical with vitamin B_{12}. Ibid., *71:* 209.

———— 1949b Protective effects of soybean meal for the immature hyperthyroid rat. J. Nutrition, *39:* 259.

ERSHOFF, B. H., AND H. J. DEUEL, JR. 1945 The effect of growth hormone on the vitamin A deficient rat. Endocrinology, *36:* 280.

ERSHOFF, B. H., AND D. HERSHBERG 1945 The beneficial effects of yeast on the cardiac failure of hyperthyroid rats. Am. J. Physiol., *145:* 16.

PERSPIRATION AS A FACTOR INFLUENCING THE REQUIREMENT FOR CALCIUM AND IRON

FRANCES A. JOHNSTON, THELMA J. McMILLAN AND ERICA R. EVANS

The College of Home Economics and the School of Nutrition, Cornell University, Ithaca, New York

(Received for publication June 5, 1950)

In studies of the iron and calcium requirement, the loss of those elements in perspiration is not usually taken into consideration. Recently Mitchell and Hamilton ('49) reported losses of approximately 2 mg of iron per liter of sweat. This loss is so large as to necessitate a large increase in the requirement whenever there is appreciable perspiration. At the American Institute of Nutrition meeting in April, 1949, exploratory studies (Johnston and Hagan, '49) made in our laboratory indicating that the loss of iron is much smaller were reported. In November, Moore ('49) stated at a symposium at the University of Illinois that work in his laboratory had failed to confirm the large losses found by the Illinois investigators. Mitchell and Hamilton ('49) found losses of calcium of 5.26 mg% in undiluted sweat during the first half hour of secretion, with a decrease to 0.4 mg% at the end of three hours. They cited 6 other investigations of the calcium content of perspiration in which values from 0.3 to 12.2 mg% were reported. Most of the values, however, fell in the neighborhood of 5 mg%.[1]

[1] Since the writing of this paper W. S. Adams, A. Leslie and M. H. Levin have published an article in Proc. Soc. Exp. Biol. Med., *74:* 46 (1950) reporting the loss of iron per liter of perspiration to be so small as to be within the limts of accuracy of the method they employed.

EXPERIMENTAL

Four college women served as subjects, each for 4 periods of approximately an hour. Previous to the perspiration period each subject remained in a tub of hot water for about one-half hour, during which time she scrubbed the surface of her skin with soapy water and a brush. The hair was wrapped in cheese-cloth to prevent the collection of perspiration from the scalp and the subject then entered the sweating chamber, in which all flat surfaces had been washed within a day preceding the experiment. After standing until perspiration appeared on the back of the hand, she was rinsed with hot tap water and then with hot distilled water, and patted dry with a clean towel. She walked on clean towels to the towel-covered platform of a scale calibrated in pounds and ounces, and her weight was recorded. She then walked on a pathway of towels to enamel pans, one of which was placed on a stool in such a way as to overhang another placed on the floor. The subject sat in one and put her feet in the other. The period was terminated when about 100 ml of perspiration had been collected. About one hour was usually required. The temperature was held at 97°F. or was raised 3 or 4°F. if the subject did not perspire readily. By boiling water in pans on hotplates the wet bulb temperature was raised to 93°F. and sometimes 2 or 3°F. higher if the dry bulb temperature had been raised. By interpretation from wet bulb and dry bulb readings the relative humidity was found to range from 74 to 90%. At the end of the perspiration period the temperature and humidity were lowered enough to stop perspiration and the subject was weighed and rinsed with warm glass-distilled water until the rinse-water was free of chlorides. Six to 8 rinsings were required. By this procedure the perspiration from all parts of the body except the scalp was collected.

The perspiration was filtered through Whatman filter paper no. 44 and frozen to preserve it for analysis. The wash-water was filtered, boiled down to a volume of 100 ml and frozen. Examination of the residues from both, under the microscope,

showed that they consisted of skin cells. There was only a very occasional particle of dark material which might have been a foreign substance. Any soluble contaminating iron or calcium, if washed into the perspiration from the surface of the skin, passed through the filter and was included in the analysis. Although stringent means were taken to cleanse the skin and to avoid contamination during the collection of perspiration, there is no way of being absolutely certain that no contamination occurred.

All the perspiration periods occurred about an hour to an hour and one-half after the subject had had lunch. At the breakfast preceding the 3rd and 4th periods each subject was given 25 mg of iron as ferric chloride. Subject V. L. had been taking every day tablets containing 5 gm of ferrous gluconate, and continued this therapy.

The perspiration was ashed in platinum dishes in a muffle-furnace at 500°C. for 16 to 18 hours. The ash was dissolved in 1:3 HCl and the solution was heated below boiling for a few minutes in the platinum dish. Iron was determined by the ortho-phenanthroline method of Saywell and Cunningham ('37). Calcium was determined by the method of McCrudden ('11) without preliminary ashing.

RESULTS

To determine the exact amount of perspiration excreted is difficult. The volume collected is much less than the volume excreted because of evaporation from the skin surface and from the surface of the liquid in the collecting vessel. An approximate weight can be found from the body-weight loss. The discrepancy between the body-weight loss and the true weight of perspiration is due to the gaseous exchange and the water evaporated from the lungs and respiratory passages. It is very small. The mean body-weight loss per hour for the 4 women of this study under temperature conditions of 97°F. dry bulb and 93°F. wet bulb was 10.87 ounces (308 gm; table 1). Different subjects lose different weights of perspiration under the same conditions. Subject Z. R.

TABLE 1

Loss of iron in perspiration

SUBJECT	PERIOD	VOLUME	TIME INTERVAL	WEIGHT LOSS	WEIGHT LOSS PER HR.	LOSS OF IRON [1]						TEMPERATURE		RELATIVE HUMIDITY
						In volume collected	In wash-water	Total	Per liter [2]	Per hour [2]	Per kg of wt. loss [2]	Dry bulb	Wet bulb	
		ml	*min.*	*oz.*	*oz.*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	°*F.*	°*F.*	%
T.M.	I	218.0	79	16.75	12.72	0.053	0.038	0.091	0.24	0.069	0.19	97	93	86
	II	273.0	55	12.75	13.91	0.062	0.026	0.088	0.23	0.096	0.24	98	93	82
	III	185.0	35	13.00	22.29	0.041	0.042	0.083	0.22	0.142	0.22	98	92–93	79–82
	IV	197.5	45	12.25	16.33	0.044	0.048	0.092	0.22	0.123	0.26	96–97	93	86–90
	Mean				16.31				0.23	0.108	0.23			
D.D.	I	120.0	75	10.00	8.00	0.027	0.038	0.065	0.22	0.052	0.23	97–101	93–97	82–88
	II	128.0	56	9.25	9.91	0.033	0.040	0.073	0.26	0.078	0.28	99	94	83
	III	109.0	62	5.00	4.84	0.026	0.036	0.062	0.24	0.060	0.44	99–101	94–95	80–83
	IV	132.5	75	16.00	12.80	0.032	0.044	0.076	0.24	0.061	0.17	100–102	93–95	76–80
	Mean				8.89				0.24	0.063	0.28			
V.L.	I	107.0	40	8.75	13.12	0.037	0.042	0.079	0.34	0.118	0.32	96–97	93–94	90
	II	80.0	70	12.00	10.29	0.020	0.030	0.050	0.25	0.043	0.15	99–101	94–96	83
	III	111.0	50	9.25	11.10	0.036	0.040	0.076	0.32	0.091	0.29	101–103	94–95	74–80
	IV	100.0	50	7.50	9.00	0.025	0.033	0.058	0.25	0.070	0.27	99–100	93	76–80
	Mean				10.88				0.29	0.080	0.26			
Z.R.	I	97.0	80	8.50	6.38	0.034	0.048	0.082	0.35	0.062	0.34	98–102	94–98	86–88
	II	57.5	70	8.00	6.86	0.026	0.031	0.057	0.45	0.049	0.25	99–101	93–94	77–83
	III	76.0	70	10.00	8.57	0.019	0.031	0.050	0.24	0.043	0.17	101	93–94	74–80
	IV	125.0	90	11.75	7.83	0.027	0.033	0.060	0.22	0.040	0.18	98–101	93–96	80–83
	Mean				7.41				0.32	0.048	0.24			
All subjects	Mean				10.87				0.27	0.075	0.25			

[1] These values do not include the loss of iron in perspiration from the scalp.

[2] Values per liter are based on undiluted perspiration; values per hour and per kilogram of weight loss are based on the sum of the losses in undiluted perspiration and in wash-water.

lost a mean of only 7.41 ounces (210 gm) per hour, while subject T. M. lost 16.31 ounces (463 gm). The same subject lost widely different weights in different periods: one subject lost 12.72 ounces (361 gm) per hour in period I and 22.29 ounces (633 gm) in period III. These differences may be accounted for partly by minor differences in the physical conditions in the perspiration chamber, but probably are due largely to differences in emotional stimuli.

The total iron loss during a perspiration period was found by adding the values for the iron content of the undiluted perspiration collected in the pans to the values for the iron in the wash-water. From the loss of body weight and the total loss of iron, the mean loss of iron per kilogram of body-weight loss for each subject was found to be 0.23, 0.24, 0.26 and 0.28 mg (table 1). The mean loss of iron per hour ranged from 0.048 mg for the subject who perspired the least to 0.108 mg (table 1) for the subject who perspired the most profusely. These values for total iron loss do not include loss from the scalp.

The mean iron content per subject for all periods for the undiluted perspiration taken from the collection pans was 0.23, 0.24, 0.29 and 0.32 mg per liter (table 1). At the same time that water was being evaporated from the perspiration while it remained on the surface of the skin, some of the salts in the perspiration were being deposited on the skin surface. Apparently the two processes balanced each other, because the iron loss in the undiluted perspiration collected in the pan was approximately the same as the loss per kilogram of weight loss (table 1).

No evidence was found that the intake of iron increased the amount excreted in the perspiration. No more iron was excreted in periods III and IV when 25 mg of iron as ferric chloride were given at breakfast than in the first two periods when no supplement of iron was added. Subject V. L., who was taking iron therapy daily, excreted no more iron than the other subjects.

The excretion of calcium in perspiration was larger than that of iron. The undiluted perspiration collected under hot humid conditions contained a mean of 1.42, 3.10, 4.38 and 4.51 mg% (table 2) for each of the 4 subjects. Subject V. L. excreted much less calcium than the other subjects; the reason for this is not known. Since both Hopf ('35) and Mitchell

TABLE 2

Loss of calcium in perspiration [1]

SUBJECT	PERIOD	LOSS OF CALCIUM [2]					
		In volume collected	In wash-water	Total	Per 100 ml [3]	Per hour [3]	Per kg of wt. loss [3]
		mg	*mg*	*mg*	*mg*	*mg*	*mg*
T.M.	I	7.24	5.72	12.96	3.32	9.84	27.23
	II	5.90	5.12	11.02	2.16	12.02	30.44
	III	7.40	5.22	12.62	4.00	21.63	34.20
	IV	5.75	4.62	10.37	2.91	13.83	29.80
	Mean				3.10	14.33	30.42
D.D.	I	7.37	5.10	12.47	6.14	9.98	43.91
	II	4.97	3.80	8.77	3.88	9.40	33.35
	III	4.52	4.42	8.94	4.15	8.65	62.96
	IV	5.14	4.03	9.17	3.88	7.34	20.20
	Mean				4.51	8.84	40.10
V.L.	I	1.28	2.20	3.48	1.20	5.22	14.03
	II	1.63	2.94	4.57	2.04	3.92	13.40
	III	1.45	2.17	3.62	1.31	4.34	13.76
	IV	1.14	1.52	2.66	1.14	3.19	12.49
	Mean				1.42	4.17	13.42
Z.R.	I	4.41	3.70	8.11	4.55	6.08	33.65
	II	3.05	5.34	8.39	5.31	7.19	36.96
	III	3.19	6.00	9.19	4.20	7.88	32.36
	IV	4.30	4.76	9.06	3.44	6.04	27.13
	Mean				4.38	6.80	32.52
All subjects	Mean				3.35	8.54	29.12

[1] Refer to table 1 for volume, time interval and weight loss.

[2] These values do not include the loss of calcium in perspiration from the scalp.

[3] Values per liter are based on undiluted perspiration; values per hour and per kilogram of weight loss are based on the sum of the loss in undiluted perspiration and in wash-water.

and Hamilton ('49) found that the calcium of perspiration decreases as sweating is continued, a logical explanation is that she had perspired each day shortly before the collection period.

The mean total loss of calcium in undiluted perspiration and washings was 13.42, 30.42, 32.52 and 40.10 mg per kilogram of weight loss (table 2), respectively, for each of the 4 subjects. On a per hour basis, the mean loss for each of the 4 subjects was 4.17, 6.80, 8.84 and 14.33 mg (table 2).

DISCUSSION

The results of this study show that the loss of iron in perspiration is small. A large excretion of iron in perspiration would not be anticipated by those who believe in the theory that little iron is absorbed by the normal, healthy individual and that once it has been absorbed the body holds onto it tenaciously. Since 1937, when McCance and Widdowson brought together the existing facts supporting this theory, much more evidence has been produced in support of it, but it has not received universal acceptance.

Losses of calcium and iron of the magnitude found in this study are such as to indicate that the intake of the average woman engaging in average activity under average conditions of temperature and humidity must be increased a little to cover them. Because the recommended allowances of the Food and Nutrition Board of the National Research Council are set high enough to allow for a factor of safety, they are probably already high enough to cover this additional requirement. Possibly for people who live in the tropics or in the temperate zone during the warm season, or for those whose work involves heavy exercise, the loss of perspiration is great enough to raise the required intake of these elements above the present "allowances." Before any estimate can be made for women under these conditions more information is needed about the amount of perspiration lost by women at various types of activity at different temperatures, and about the changes in the mineral content of

perspiration at different rates of secretion, over prolonged periods and after acclimatization to high temperatures. As has already been stated, both Hopf ('35) and Mitchell and Hamilton ('49) found that the calcium content of perspiration decreased as the duration of the sweating period was lengthened. This points to the possibility that calcium losses may also decrease with acclimatization. Any estimate of the loss of minerals in perspiration is complicated by unpredictable increases in the volume of perspiration due to emotional stimuli.

The subjects of this study lost less perspiration per hour than did those of Mitchell and Hamilton ('49) under similar conditions of temperature and humidity. The mean loss for the 4 women of this study was 10.87 ounces (308 gm) per hour, which was less than half the 720 gm which was the mean loss for the 4 men of their study. This difference may be partly due to differences in physical conditions other than temperature and humidity, to the possibility that the subjects of this study were less active — although the subjects of both studies sat during the collection periods — or to a difference in mental attitude, since emotional stimuli can cause a great increase in secretion. The possibility is suggested that men perspire more profusely than women under identical conditions.

The loss of iron in perspiration found in this study is much less than that found by Mitchell and Hamilton. They found losses of iron of about 2 mg per kilogram of body-weight loss and of 1.29 mg per hour; the corresponding losses in this study were 0.25 mg and 0.075 mg.

The reason for the differences in the two studies is not known. Five possibilities are suggested:

1. Some of the iron in the perspiration may have been lost or remained undetected by the method of analysis used in this laboratory. Before the analyses of these samples were begun, much time was spent on studying the analytical method to be employed. The perspiration was ashed by both wet and dry methods. Both the thiocyanate and the ortho-

phenanthroline methods for iron determination were used. Despite the fact that perspiration contains little or no phosphorus, some dry-ashed samples were given an acid hydrolysis to convert pyrophosphates to orthophosphates. As this process did not increase the yield of iron, it was abandoned. The method finally evolved has already been described. A sample of the perspiration was analyzed in another laboratory where analyses of biological materials were being made routinely; the resulting values agreed with ours.

2. Iron from sources other than perspiration may have been included in the values reported by Mitchell and Hamilton. In a private communication Dr. Mitchell supplied the following information pertaining to this point:

"The possibility of iron contamination of the body surfaces of our subjects was always kept in mind and all conceivable precautions were taken to avoid it. The fact that the iron content of the collected sweat was not demonstrably affected by the rate of sweating offers objective evidence that the measures taken were successful, since one would not expect the contamination to vary with the rate of sweating. For sweat specimens produced at rates of less than 200 ml per hour, the iron content, based on net loss in body weight, averaged 1.91 mg per liter, and for sweat produced at rates of 200 to 900 ml per hour, the iron content averaged 1.64 mg per liter. The t value of the difference (Fisher, 1949 ed.) is 1.464, and the probability that a random combination of the uncontrolled factors in the experiment could have produced a mean difference as large or larger than that observed is 0.17. This probability is too large to be disregarded. Hence, there is no compelling reason to believe that rate of sweating was a determining factor in the concentration of iron in sweat."

3. The difference in sex of the subjects in the two experiments may be a factor. This may be due to a difference in sweat glands. The apocrine glands, restricted mainly to areas where hair follicles occur, contain inorganic, stainable

iron in the segments of the glands (Bunting, Wislocki and Dempsey, '48), while the eccrine glands, distributed over the entire body, do not. Possibly the apocrine glands secrete iron and the eccrine glands do not; possibly the secretion collected from the men contained a higher proportion of perspiration secreted by the apocrine glands.

4. A difference in the completeness of collection may have influenced the results. While Mitchell and Hamilton collected sweat from all parts of the body, the collections in this laboratory did not include the sweat from the scalp. That sweating occurs on the scalp has been shown by Weiner ('45). Since the apocrine glands are associated with hair follicles, the perspiration from that area may be comparatively rich in iron. Dr. Mitchell states that: "The necessity of washing all of the body thoroughly in collecting sweat for iron analysis is revealed by the results secured at Illinois when undiluted sweat was collected on restricted skin areas mainly on the ventral surface of the body. In separate specimens, the values secured in micrograms of Fe per 100 ml of undiluted sweat ranged from 12 to 177."

5. A part of the difference in the values for the loss of iron per hour is due to the fact that the men serving as subjects in the Illinois study lost much more perspiration per hour than did the women of the present study.

For calcium Mitchell and Hamilton found a loss of 2.94 mg% in first samples of undiluted persipration in one experiment, and in another a loss of 5.26 mg% in the first half hour and 3.17 in the second half hour. They found a total loss in undiluted perspiration and wash-water of 26 mg per kilogram of body-weight loss and 20.2 mg per hour on a normal intake. The corresponding losses found in this study were 3.35 mg%, 29.12 mg per kilogram and 8.54 mg per hour. The loss of calcium in the two studies is similar except for the hourly loss. This would be anticipated because the subjects of this study lost much less perspiration per hour.

SUMMARY

Perspiration was collected from 4 young women, each of whom served as a subject for 4 periods of approximately an hour each. The temperatures used were 97°F. dry bulb and 93°F. wet bulb. The collection was made in enamel tubs in which the subjects sat during the sweating period. Perspiration from all parts of the body except the scalp was collected.

The mean loss of body-weight per hour during the collection periods was 10.87 ounces (308 gm). The mean loss of iron per kilogram of body-weight loss for each of the 4 subjects was 0.24, 0.26, 0.26, and 0.28 mg; the mean loss per hour was 0.48, 0.063, 0.080 and 0.108 mg. The mean loss of calcium per kilogram of body-weight loss for each of the 4 subjects was 13.42, 30.42, 32.52 and 40.10 mg; the mean loss per hour was 4.17, 6.80, 8.84 and 14.33 mg.

The administration of 25 mg of iron with breakfast on the day of the collection did not increase the loss of iron, nor did a subject who was taking iron therapy daily secrete more iron than the other subjects.

LITERATURE CITED

BUNTING, H., G. B. WISLOCKI AND E. W. DEMPSEY 1948 The chemical histology of human eccrine and apocrine sweat glands. Anat. Rec., *100:* 61–78.

HOPF, G. 1935 Chemisch-physikalische Untersuchungen über den menschlichen Schweiss. Arch. Derm. Syph., *171:* 301–312.

JOHNSTON, F. A., AND G. C. HAGAN 1949 Perspiration as a factor influencing the iron requirement. Fed. Proc., *8:* 387.

MCCANCE, R. A., AND E. M. WIDDOWSON 1937 The absorption and excretion of iron. Lancet, *2:* 680–684.

MCCRUDDEN, F. H. 1911 The determination of calcium in the presence of magnesium and phosphates: the determination of calcium in urine. J. Biol. Chem., *10:* 187–199.

MITCHELL, H. H., AND T. S. HAMILTON 1949 The dermal excretion under controlled environmental conditions of nitrogen and minerals in human subjects, with particular reference to calcium and iron. Ibid., *178:* 345–361.

MOORE, C. V. 1949 Iron metabolism and hypochromic anemia. Read at a symposium on "Currents in Nutrition," Univ. of Ill. College of Med., Chicago, November 19, 1949.

SAYWELL, L. G., AND B. B. CUNNINGHAM 1937 Determination of Iron. Ind. Eng. Chem., Anal. Ed., *9:* 67–69.

WEINER, J. S. 1945 The regional distribution of sweating. J. Physiol., *104:* 32–40.

UTILIZATION OF DEHYDROASCORBIC ACID BY HUMAN SUBJECTS[1]

E. NEIGE TODHUNTER, THELMA McMILLAN[2] AND DOROTHY A. EHMKE

Research Laboratory of Human Nutrition, University of Alabama, Tuscaloosa

(Received for publication April 14, 1950)

The antiscorbutic activity of dehydroascorbic acid has been reported (Fox and Levy, '36), but the extent of the utilization of dehydroascorbic acid has not been clearly established. Roe and Barnum ('36), on the basis of oral and subcutaneous administration to guinea pigs, reported that dehydroascorbic acid had one-fourth the potency of reduced ascorbic acid; other investigators using guinea pigs have reported 80% (Gould and Shwachman, '43) and 100% (Penny and Zilva, '43). With human subjects, Borsook et al. ('37) found that orange juice treated with charcoal maintained plasma ascorbic acid at the same level as fresh orange juice.

Further data on human utilization are needed, particularly since the total ascorbic acid in foods is now commonly reported and used in diet calculations.

The present experiment was designed to ascertain the availability of dehydroascorbic acid as a source of vitamin C for the human subject. The plan of the experiment was to stabilize the ascorbic acid content of plasma and urine at the level of ascorbic acid intake recommended by the National Research Council, and then to observe the effect when orange

[1] Supported by grants from the General Education Board and the Research Committee of the University of Alabama.

[2] Present address: Graduate Research Assistant in Nutrition, Cornell University, Ithaca, N. Y.

juice as a source of reduced ascorbic acid was replaced by norit-treated orange juice as a source of dehydroascorbic acid.

METHODS

The experiment was divided into three periods:

Period I

The preliminary stabilization period lasted 7 days and was intended to bring the ascorbic acid in the blood serum of each subject to a constant level. During this time the subjects continued on their customary diets except that all fruits and vegetables were omitted and 75 mg of ascorbic acid were given daily in tablet form. This preliminary period was started at the end of the menstrual period for each subject.

Period II

The control period followed immediately and lasted 5 days. Subjects were given a basal diet adequate in all nutrients except vitamin C. The basal diet included servings of fruits and vegetables low in vitamin C; these were added to increase the palatability of the diet and the satisfaction of the subject. The basal diet supplied 7.7 mg of reduced ascorbic acid and was supplemented daily with 65 mg of reduced ascorbic acid in the form of orange juice.

Period III

The test period followed immediately and lasted 5 days. Subjects received the same basal diet as in period II and a daily supplement of 65 mg of dehydroascorbic acid prepared from orange juice. The experiment was repeated for each subject immediately following the end of the intervening menstrual period. In this second experiment the sequence of the control and test periods was reversed.

Subjects

Six women students served as subjects during the first year of the study. The experiments were repeated the next year with two subjects, one of whom had participated in the first series of experiments, thus giving opportunity for further study of the experimental reactions of this individual.

The subjects met together prior to the experiment and discussed all phases of the investigation, so that there was complete understanding and fullest cooperation throughout. Specific instructions on all procedures were typed and given to each subject for each period.

Subjects were all between 20 and 30 years of age. They were weighed at the beginning and end of the experiment and all had maintained constant weight during the experimental period. Individual data were as follows:

Subject	Age	Height — inches	Weight — lb.
A	28	63¾	156
B	20	70½	148
C	23	63¾	112
D	21	65½	118
E	20	61¼	129
F	30	67	120
G	22	69¾	143

Collection and analysis of urine

Urine was collected in wide-mouthed quart-size preserving jars with glass tops. Small wooden boxes were provided for carrying the jars and served also to exclude light. A sufficient quantity of 50% metaphosphoric acid was added to each jar to give a final concentration in the urine of 6%. Urine voided during the day was stored in a refrigerator in the laboratory; that voided at night was stored in as cool a place as possible and brought to the laboratory before breakfast. Twenty-four-hour samples were analyzed each morning for reduced ascorbic acid and total ascorbic acid.

Reduced ascorbic acid was determined by the method of Bessey ('38) using the Coleman spectrophotometer, model 11. Total ascorbic acid was determined by the method of Roe and Kuether ('43).

Urine was collected daily throughout all periods, beginning with the 5th day of the stabilization period.

Collection and analysis of blood

Blood samples were taken daily before breakfast, beginning with the 5th day of the stabilization period. Capillary blood was collected by finger prick in 4 capillary tubes, which were then sealed and centrifuged for 10 minutes. Reduced ascorbic acid was determined by the micro-method of Mindlin and Butler ('38) with the quantities of reagents adjusted to 0.02 ml serum. Readings were made in a Coleman spectrophotometer (model 6) using micro-absorption tubes. The total ascorbic acid content of the serum was determined by the method of Roe and Kuether as modified by Lowery, Lopez and Bessey ('45) for 0.025 ml serum.

Basal diet and supplements

Menus were planned for 5 days and were of constant composition with regard to protein and minerals but allowed some variety each day in the source of protein and the method of preparation of certain foods. Each day's menu provided (by calculation) 63 gm of protein; one pint of milk daily was allowed. In order to ensure adequate vitamin intakes, supplements of vitamins A and D were taken each morning at breakfast and a supplement of the vitamin B complex was taken at the noon meal. Canned peas, carrots or green beans were served as the vegetable and canned plums or applesauce as the fruit. A sufficient quantity of the same brand and pack of each fruit and vegetable was purchased to last throughout the study. These products were analyzed for reduced ascorbic acid by the method of Loeffler and Ponting ('42). The same amount of each fruit or vegetable was fed throughout the

study to provide, along with the milk, a total of 7.7 mg of reduced ascorbic acid in the basal diet. Each subject satisfied her calorie needs with additional breadstuff as desired.

Menus were rotated in the same order in each experimental period. A sample menu was: *Breakfast:* cereal, egg, toast, butter, coffee; *lunch:* ground beef pattie (100 gm), rice and gravy, green beans (125 gm), bread, chocolate pudding, milk; *supper:* cheese (45 gm) sandwich, canned pears (150 gm), cookie, milk.

In the control period 65 mg of reduced ascorbic acid in the form of orange juice were taken at the evening meal. Canned orange juice of the same brand and pack was purchased in sufficient quantity for use throughout the study.

Preparation of dehydroascorbic acid

Dehydroascorbic acid was prepared from canned orange juice by treatment with norit; norit from the same sample was used throughout. In preliminary experiments, it was found that 4 gm of norit added to 110 ml of orange juice gave best results. Orange juice before treatment had 47.13 mg of reduced ascorbic acid per 100 ml of juice, and after norit treatment 46.31 mg of dehydroascorbic acid per 100 ml of juice were obtained. Dehydroascorbic acid was freshly prepared each afternoon and taken with the evening meal; 65 mg of dehydroascorbic acid were taken daily by each subject during the test period.

RESULTS AND DISCUSSION

Reduced ascorbic acid in blood and urine

The amounts of reduced ascorbic acid present in the blood serum and urine of all subjects are shown in table 1.

There was considerable variation from day to day between individuals, and for each individual, in all periods, although diet and ascorbic acid intake were constant during the period. There was some indication that body weight may have been an influencing factor; the highest initial serum level was for sub-

TABLE 1

Reduced ascorbic acid in blood serum and urine for subjects receiving 65 mg ascorbic acid in reduced or dehydro-form (milligrams per 100 ml, means and standard deviations)

PERIOD	SUBJECT: A	B	C	D	E	F_1	F_2	G
				Blood				
Test I								
Stabilization	1.16 ± 0.01	0.70 ± 0.18	1.44 ± 0.20	1.30 ± 0.26	1.11 ± 0.12	1.46 ± 0.07	1.18 ± 0.10	0.94 ± 0.11
Ascorbic acid	1.10 ± 0.15	0.85 ± 0.12	1.44 ± 0.19	1.20 ± 0.14	1.26 ± 0.05	1.14 ± 0.13	1.04 ± 0.08	0.87 ± 0.06
Dehydroascorbic acid	0.77 ± 0.13	0.60 ± 0.03	1.25 ± 0.07	0.89 ± 0.09	1.19 ± 0.10	1.08 ± 0.10	0.89 ± 0.08	0.71 ± 0.10
Test II								
Stabilization	1.10 ± 0.11	0.87 ± 0.10	1.46 ± 0.09	1.24 ± 0.07	1.21 ± 0.14	1.32 ± 0.07	1.05 ± 0.15	0.85 ± 0.10
Dehydroascorbic acid	1.01 ± 0.19	0.75 ± 0.14	1.31 ± 0.12	0.97 ± 0.14	1.09 ± 0.16	0.98 ± 0.13	0.89 ± 0.21	0.67 ± 0.06
Ascorbic acid	0.87 ± 0.16	0.68 ± 0.24	1.20 ± 0.10	1.18 ± 0.07	1.24 ± 0.11	1.15 ± 0.08	1.07 ± 0.04	0.86 ± 0.11
				Urine				
Test I								
Stabilization [1]	19.3	31.1	17.8	20.9	29.7	14.2	17.1	23.8
Ascorbic acid	23.8 ± 4.37	30.3 ± 6.42	18.4 ± 3.13	24.6 ± 9.96	20.7 ± 3.64	29.7 ± 9.25	18.2 ± 7.22	24.4 ± 4.58
Dehydroascorbic acid	13.9 ± 2.38	16.0 ± 2.82	7.0 ± 2.72	17.1 ± 2.41	14.8 ± 3.31	14.5 ± 1.28	13.6 ± 1.87	14.8 ± 5.10
Test II								
Stabilization [1]	22.8	34.5	26.3	18.6	21.0	22.0	36.9	25.0
Dehydroascorbic acid	17.6 ± 5.26	28.6 ± 8.58	8.4 ± 3.41	14.1 ± 5.19	13.3 ± 8.81	18.5 ± 9.71	17.3 ± 4.83	21.3 ± 8.01
Ascorbic acid	13.2 ± 1.36	20.4 ± 5.38	7.8 ± 1.38	14.0 ± 3.88	12.8 ± 4.52	13.7 ± 1.48	20.2 ± 7.77	14.3 ± 6.05

[1] Analyses were for the last three days of this period.

ject C, who weighed 112 lb., and the lower level of 0.70 mg per 100 ml was for subject B, weighing 148 lb.

In test I, when ascorbic acid was fed, subjects C and E had blood serum levels equal to or above those for the preliminary stabilization period; for all other subjects except F_1 the ascorbic acid in the serum was not significantly different [3] in the two periods.

When the dehydroascorbic acid was given, the blood levels for all subjects were lower, and for all except E and F_1 the difference was significant. Similarly, when ascorbic acid was fed the urinary excretion was equal to or above that of the stabilization period for all subjects except E, but when dehydroascorbic acid was given there was a significantly lower excretion of reduced ascorbic acid for all subjects except D and F_2.

In test II, when dehydroascorbic acid was fed immediately following the preliminary stabilization period, blood and urine levels were decreased significantly for 4 subjects. When ascorbic acid was given, the blood levels increased again for 5 subjects but in only three cases did they equal those of the stabilization period. Urinary excretion for 4 subjects remained approximately the same as in the preceding period.

The mean differences between periods and the significant ratio (S. R.) are shown in table 2.

Relation between ascorbic acid in blood and urine

There was no direct relationship between the ascorbic acid content of blood and the amount excreted in the urine in the stabilization period or when orange juice was given; high excretions were obtained for high (subject E) and for low (subject B) blood levels and low excretions (subject C) for

[3] Significance was determined from the difference of means divided by the square root of the sum of the squares of the standard errors; a difference of means is usually considered statistically significant if the ratio is two or greater than two.

high blood levels. However, when the blood level decreased with the feedings of dehydroascorbic acid the urinary excretion also decreased for all subjects.

Comparison of results obtained by phenylhydrazine and indophenol methods

In table 3 data are presented for blood and urine analyzed by the phenylhydrazine method of Roe and Kuether ('43), which measures the total ascorbic acid present.

If ascorbic acid were the only constituent of blood and urine being determined by these two methods (indophenol vs. phenylhydrazine), the results should be the same, but if dehydroascorbic acid were present in the urine or blood, the values obtained by the phenylhydrazine method would be ex-

TABLE 2

Difference and probable significance of difference[1] between mean values for each subject for ascorbic acid and dehydroascorbic acid period in test I and stabilization and dehydroascorbic acid period in test II

SUBJECT	TEST I				TEST II		
	Blood		Urine		Blood		Urine
	Diff.	S R[2]	Diff.	S R	Diff.	S R	Diff.[3]
A	0.33	3.0	9.9	3.1	0.09	0.9	5.2
B	0.25	4.0	14.3	4.2	0.12	1.4	5.9
C	0.19	2.0	11.4	5.5	0.15	2.0	17.9
D	0.31	6.0	7.5	1.5	0.27	3.4	4.5
E	0.07	1.5	5.9	2.4	0.12	1.1	7.7
F_1	0.06	0.9	15.2	3.3	0.34	4.7	3.5
F_2	0.15	3.0	4.6	1.3	0.16	1.2	19.6
G	0.16	2.7	9.6	2.9	0.18	3.0	3.7

[1] Significance was determined from the difference of means divided by the square root of the sum of the squares of the standard errors.

[2] S R = significance ratio. To be statistically significant this value should be two or greater than two.

[3] Only three days' urine analyzed in stabilization period, therefore data not treated statistically.

TABLE 3

Total ascorbic acid in blood serum and urine for subjects receiving 65 mg ascorbic acid in reduced or dehydro-form (milligrams per 100 ml, means and standard deviations)

PERIOD	SUBJECT: A	B	C	D	E	F_1	F_2	G
				Blood				
Test I								
Stabilization	1.26 ± 0.07	0.80 ± 0.19	1.54 ± 0.14	1.15 ± 0.07	1.17 ± 0.09	1.31 ± 0.05	1.43 ± 0.16	1.25 ± 0.13
Ascorbic acid	1.07 ± 0.15	0.83 ± 0.07	1.37 ± 0.16	1.05 ± 0.09	1.13 ± 0.07	1.06 ± 0.06	1.20 ± 0.03	1.02 ± 0.07
Dehydro-ascorbic acid	0.88 ± 0.13	0.62 ± 0.05	1.21 ± 0.02	0.88 ± 0.11	1.11 ± 0.13	0.92 ± 0.06	1.02 ± 0.06	0.81 ± 0.02
Test II								
Stabilization	0.93 ± 0.02	0.67 ± 0.02	1.17 ± 0.11	0.97 ± 0.03	0.99 ± 0.10	1.07 ± 0.10	1.23 ± 0.09	0.93 ± 0.03
Dehydro-ascorbic acid	0.84 ± 0.05	0.55 ± 0.07	0.89 ± 0.19	0.81 ± 0.06	0.97 ± 0.09	0.90 ± 0.07	1.03 ± 0.13	0.79 ± 0.11
Ascorbic acid	0.75 ± 0.16	0.54 ± 0.07	1.12 ± 0.10	0.82 ± 0.12	0.95 ± 0.05	0.86 ± 0.05	1.16 ± 0.04	0.85 ± 0.07
				Urine				
Test I								
Stabilization[1]	25.2	37.8	28.5	28.4	35.5	18.6	17.0	25.7
Ascorbic acid	22.8 ± 5.78	35.6 ± 12.76	20.6 ± 6.45	28.1 ± 14.45	20.1 ± 5.88	35.6 ± 15.23	19.2 ± 5.62	26.3 ± 5.56
Dehydro-ascorbic acid	11.2 ± 1.43	18.2 ± 4.65	9.7 ± 2.67	15.1 ± 1.66	15.3 ± 2.54	15.9 ± 4.16	17.2 ± 5.01	17.7 ± 3.79
Test II								
Stabilization[1]	25.4	41.4	33.8	27.1	31.7	30.1	34.4	28.7
Dehydro-ascorbic acid	16.9 ± 4.83	35.9 ± 12.76	12.9 ± 5.87	21.5 ± 5.65	16.7 ± 9.81	24.6 ± 13.00	17.9 ± 6.37	20.6 ± 4.38
Ascorbic acid	15.0 ± 3.29	25.6 ± 8.13	10.9 ± 2.92	14.4 ± 4.61	15.7 ± 3.11	14.6 ± 2.77	25.0 ± 9.40	17.0 ± 6.26

[1] Analyses were for the last three days of this period.

pected to be higher. That this was not the case for blood is seen by comparison of the mean values presented in tables 1 and 3. In test I in the analyses of serum there were an equal number of instances in which the phenylhydrazine method gave values above and below that obtained by the indophenol method, but the mean of all differences was 0.20 mg higher by the phenylhydrazine method. In test II only 5 of the mean blood values were higher by the phenylhydrazine method, and the mean of all differences was 0.13 mg lower by this method. The higher values by the phenylhydrazine method did not occur consistently during the period when dehydroascorbic acid was being fed.

Kellie and Zilva ('36) reported that if the erythrocytes are intact the blood is incapable of dehydrogenating ascorbic acid *in vivo* and that the animal organism tends to keep the vitamins in the reduced state; if hemolysis occurs, dehydroascorbic acid is formed. Roe and Barnum ('36) reported the presence in blood of a substance believed to be an enzyme which had the ability to reduce ascorbic acid in the blood of humans, guinea pigs, and rats. Borsook and co-workers ('37) could find no evidence of such an enzyme.

Analysis of the urine by the phenylhydrazine method gave higher values 19 times in test I and 21 times in test II; the mean of all differences by the two methods was 2.7 mg higher in test I and 4.0 mg higher in test II for the phenylhydrazine method. Berryman and co-workers ('44) have also reported higher values by the Roe and Kuether ('43) method, and for a group of 68 men found that an average of 18% of the total excretion was dehydroascorbic acid.

In the present studies there was for a few subjects an appreciable difference in the values obtained by the two methods, but there was no consistent evidence of the presence of dehydroascorbic acid in blood or urine. The effects of feeding dehydroascorbic acid were the same whether reduced or total ascorbic acid was measured.

SUMMARY

The reduced and total ascorbic acid contents of the blood and urine of 7 healthy young women were determined for periods on a low-vitamin-C basal diet with daily supplements of: (a) orange juice containing 65 mg of reduced ascorbic acid or (b), norit-treated orange juice containing 65 mg of dehydroascorbic acid.

When dehydroascorbic acid was fed, the levels of reduced ascorbic acid in blood serum and in urine were comparable to those obtained when the reduced form of the vitamin was given, indicating satisfactory utilization of dehydroascorbic acid.

LITERATURE CITED

BERRYMAN, G. H., C. E. FRENCH, H. A. HARPER AND H. POLLACK 1944 Response to the intravenous injection of ascorbic acid as indicated by the urinary excretion of the total and reduced forms. J. Nutrition, *27:* 309.

BESSEY, O. A. 1938 A method for determination of small quantities of ascorbic acid and dehydroascorbic acid in turbid and colored solutions in the presence of other reducing substances. J. Biol. Chem., *126:* 771.

BORSOOK, H., H. W. DAVENPORT, C. E. P. JEFFREYS AND R. C. WARNER 1937 The oxidation of ascorbic acid and its reduction *in vitro* and *in vivo*. Ibid., *117:* 237.

FOX, F. W., AND L. F. LEVY 1936 Experiments confirming the antiscorbutic activity of dehydroascorbic acid and a study of its storage and that of ascorbic acid by the guinea pig at different levels of intake. Biochem. J., *30:* 211.

GOULD, B. S., AND H. SHWACHMAN 1943 A new method for bioassay of antiscorbutic substances. Assays of dehydroascorbic acid, 2-ketogulonic acid, iron ascorbate, and the effectiveness of oral and parenteral administration of ascorbic acid. J. Biol. Chem., *151:* 439.

KELLIE, A. E., AND S. S. ZILVA 1936 The alleged presence of dehydroascorbic acid in blood. Biochem. J., *30:* 361.

LOEFFLER, H. J., AND J. D. PONTING 1942 Ascorbic acid. Rapid determination in fresh, frozen or dehydrated fruits and vegetables. Ind. Eng. Chem., Anal. Ed., *14:* 846.

LOWERY, O. H., J. A. LOPEZ AND O. A. BESSEY 1945 The determination of ascorbic acid in small amounts of blood serum. J. Biol. Chem., *160:* 609.

MINDLIN, R. L., AND A. M. BUTLER 1938 The determination of ascorbic acid in plasma; a macromethod and micromethod. Ibid., *122:* 673.

PENNY, J. R., AND S. S. ZILVA 1943 The chemical behavior of dehydro-l-ascorbic acid *in vitro* and *in vivo*. Biochem. J., *37:* 403.

ROE, J. H., AND G. L. BARNUM 1936 The antiscorbutic potency of reversibly oxidized ascorbic acid and the observation of an enzyme in blood which reduces the reversibly oxidized vitamin. J. Nutrition, *11:* 359.

ROE, J. H., AND C. A. KUETHER 1943 The determination of ascorbic acid in whole blood and urine through the 2, 4-dinitrophenylhydrazine derivative of dehydroascorbic acid. J. Biol. Chem., *147:* 399.

THE VITAMIN E REQUIREMENT OF GUINEA PIGS [1]

FLORENCE A. FARMER, BARBARA C. MUTCH,[2] J. M. BELL,[3]
L. D. WOOLSEY [4] AND E. W. CRAMPTON
Department of Nutrition, Macdonald College, Quebec, Canada

(Received for publication July 3, 1950)

INTRODUCTION

A recent review of the literature relating to the vitamin E requirement of guinea pigs, by Mannering ('49), shows that vitamin E deficiency has been observed frequently but that the exact requirement of this species is not known. Shimotori et al. ('39) showed that 1.5 mg of pure alpha-tocopherol per day was sufficient to protect guinea pigs from muscular dystrophy for as long as 200 days. Pappenheimer and Goettsch ('41) found weekly doses of 5 or 10 mg sufficient to prevent muscular dystrophy in 5 pregnant guinea pigs but inadequate for protection against resorption of the fetuses. Higher doses were not used. The basal diet in this study was composed of casein, sucrose, cornstarch, yeast, salt, lard, cellulose and about 2% cod liver oil. To this were added 5 ml tomato juice and 25 gm fresh lettuce daily.

In the course of an effort to find a satisfactory diet for use in the bioassay of vitamin C with guinea pigs, an oppor-

[1] This investigation was supported in part by the Department of National Health and Welfare (formerly the Department of Pensions and National Health) and the National Research Council of Canada.

[2] Now at the Institute of Nutrition, University of Montreal, Canada.

[3] Now at the Department of Animal Husbandry, University of Saskatchewan, Canada.

[4] Now at the Dominion Chinchilla Ranchers, Ltd., Napanee, Canada.

tunity was afforded of studying the vitamin E requirement of that species. Several different diets were fed to young growing animals and also to pregnant females.

EXPERIMENTAL

Test with young, growing guinea pigs

Fifteen male weanling guinea pigs were fed a vitamin E-low diet ad libitum and given a weekly dose of cod liver oil. The composition of the basal diet (basal diet 6) is given in table 1. After 8 weeks the animals were killed and examined. Three of the 15 showed skeletal muscle degeneration. In one case the condition was classified as severe.

In 1943 a vitamin C bioassay was performed in this laboratory involving 192 guinea pigs on several different levels of ascorbic acid. The experiment was designed in such a way

TABLE 1

Percentage composition of Macdonald guinea pig basal diets

INGREDIENT	BASAL DIET NUMBER					
	1	2	3[1]	4[2]	5[2]	6[2]
Oats	..	..	15	15	15	15
Wheat	7.5	9	10	10.5	13	13
Barley	15.5	18	..	..	..	..
Soybean oilmeal	10	10	..	..	..	..
Linseed oilmeal	..	..	10	10	12.5	12.5
Skim milk powder	..	..	15	15	15	15
Bone char	2	2	4	4	4	4
Iodized salt	0.5	0.5	0.5	0.5	0.5	0.5
Wheat bran	7	7	..	..	..	..
Beet pulp	20	26	25	25	25	20
Fish meal	12	12	5	5	5	5
Brewers' yeast	5	5	10	10	10	10
Wheat germ	10	10	5	5	..	..
Feeding fish oil (in diet)	0.5	0.5	0.5	..	..	..
Molasses	..	..	..	..	..	5
Corn oil	10	..	..	..	..	..

[1] Alpha-tocopherol given directly into mouths of young growing guinea pigs but none given to pregnant females.

[2] Alpha-tocopherol and cod liver oil given directly into mouths of guinea pigs on alternate days.

that the effect of vitamin E at all levels of ascorbic acid could be tested.

The vitamin E, a solution of 6 mg of alpha-tocopherol[5] in 0.4 ml of corn oil, was fed to half the animals every other day. On the alternate days vitamin A was fed to half the animals in each group. Since the latter had no bearing on the results of the experiment, it will not be considered in the discussion.

The composition of the basal diet used (basal diet 3) is shown in table 1. In this diet 5% wheat germ was added

TABLE 2

The influence of alpha-tocopherol on the incidence of muscle degeneration in young growing guinea pigs

VITAMIN SUPPLEMENTS		INITIAL WEIGHT	AVERAGE DAILY GAIN, 8 WEEKS	% SHOWING SKELETAL MUSCLE DEGENERATION		
Ascorbic acid	Alpha-tocopherol			Marked	Slight	Absent
mg/day	*mg/day*	*gm*	*gm*	%	%	%
1	3	312	4.6	0	0	100
2	3	297	5.4	0	0	100
4	3	302	6.0	0	0	100
8	3	294	5.6	0	0	100
1	0	304	4.7	63	37	0
2	0	319	5.8	19	19	62
4	0	282	5.9	50	25	25
8	0	293	5.9	31	19	50

to supply vitamin E. The cod liver oil was mixed into the diet. Madsen et al. ('35) showed that it is undesirable to mix cod liver oil into the diet of herbivora. However, according to Shimotori et al. ('40), 5% wheat germ should have prevented the dystrophy of vitamin E deficiency caused by the inclusion of cod liver oil in the diet. The results of the following experiment do not confirm Shimotori's findings.

At the end of an 8-week period the guinea pigs were killed with chloroform and given post mortem examinations. Ninety-three per cent of the animals on the 0.25-mg level of vitamin

[5] Alpha-tocopherol was supplied by Merck and Company, Montreal, Canada.

C and 44% of those on the 0.5-mg level showed hemorrhages around the knee joints due to scurvy. In these animals the muscle changes due to vitamin E deficiency could not be seen. The results presented, therefore, are based on the 128 animals which received 1 to 8 mg ascorbic acid per day. The incidence of degeneration of skeletal muscle is shown in table 2.

The results presented in this table show that alpha-tocopherol had no effect on weight, whereas it completely prevented skeletal muscle degeneration. The high incidence of skeletal muscle degeneration among animals on this basal diet, unsupplemented, is evidence that this diet was also low in vitamin E.

Tests with mature female guinea pigs

Running parallel with this work on the bioassay of vitamin C was a study of the dietary requirements of pregnant guinea pigs. Several different basal diets were used, some of which included cod liver oil. In other cases the cod liver oil was placed directly into the mouths of the animals. Table 1 shows the percentage composition of the basal diets used in this study.

These basal diets were fed to 294 pregnant guinea pigs. Some of the animals received approximately 100 gm of green feed per day (fresh lawn clippings in the summer and cabbage in the winter) in addition to the basal diet. Others, fed the basal diet alone, acted as controls. The cod liver oil was mixed into basal diets 1, 2 and 3. All females on basal diet 4 and multipara on basal diet 5 received 0.5 ml cod liver oil and 6 mg alpha-tocopherol on alternate days, given directly into the mouth. Primipara on basal diet 5 received the cod liver oil and 12 mg alpha-tocopherol every other day. Basal diet 6 was not used for pregnant animals.

At the end of each pregnancy the animals were returned to the stock colony to be remated. For this reason post mortem examinations were not always possible. However, all animals which died during the test were examined; in no

case was skeletal muscle degeneration seen. It should be noted that all these animals were multipara. The muscle degeneration seen in the smooth muscles of the stomach and intestines is thought not to be related to the absence of vitamin E, since this condition is never seen in young animals, even those showing severe degeneration of skeletal muscle.

Table 3 shows the effect of diet on the outcome of pregnancy with respect to litters (at least one living young); abortions (including litters in which all young were born dead); hemorrhage (accompanied in most cases by resorption); and the death of the mothers. It may be seen in this table that primipara are better able to withstand nutritional deficiencies than are multipara. Primipara in this study included young females mated as soon as they reached 500 gm in weight. Multipara included older females which had had at least one litter previously. Any comparison between diets must therefore be made within these groups. With this in mind, and considering only diets without green feed, we can compare basal diet 1 with basal diets 4 and 5. Only two young animals were used on basal diet 1 and both of these had received this same diet from weaning. Their mothers, however, had received green feed during pregnancy. The 10 young animals on basal diet 4 had received the same diet from weaning but in this case their mothers had also been on basal diet 4 throughout their pregnancy. In spite of this, females receiving basal diet 4 were far superior to those on basal diet 1. As this almost completely rules out the possibility of any storage effect, it is fair to say that basal diet 4 is better for young pregnant guinea pigs than is basal diet 1.

By the same standard, basal diet 5 should have given at least as good results as basal diet 4, since these young females were born to mothers receiving grass. In spite of this, 5% of the 20 pregnancies resulted in failure to produce young. Thus we conclude that basal diet 4 is superior to basal diet 5 in supplying the nutritional requirements of young pregnant guinea pigs. Similarly, a comparison of the

TABLE 3

The effect of diet upon the outcome of pregnancy in primiparous and multiparous guinea pigs

BASAL DIET NO.	METHOD OF ADMINISTERING COD LIVER OIL	DAILY AMOUNTS OF SUPPLEMENTS		PREGNANT FEMALES		PREGNANCIES TERMINATING IN HEMORRHAGE AND ABORTION	PER CENT DYING	PER CENT LITTERS (AT LEAST ONE LIVING YOUNG)
		Green feed	Vitamin E	Number	Age [1]			
		gm	*mg*			%	%	%
1	in diet	0	0	9	m	100	0	0
1	in diet	0	0	2	p	100	0	0
2	in diet	0	0	70	m	59	11	30
3	in diet	0	0	5	m	60	40	0
4	dosed	0	3	80	m	22	12	66
4	dosed	0	3	10	p	0	0	100
5	dosed	0	3	18	m	50	0	50
5	dosed	0	6	20	p	5	0	95
2	in diet	100	0	25	m	16	0	84
3	in diet	100	0	5	m	20	0	80
4	dosed	100	3	10	p	0	0	100
5	dosed	100	3	20	m	10	0	90
5	dosed	100	6	20	p	0	0	100

[1] m — multipara; p — primipara.

diets for the older animals can be made. A comparison of the percentage of litters produced places the 5 diets in the following order of decreasing value: 4, 5, 2, 3 and 1. This, in so far as the comparison is valid, agrees well with the conclusions based on data from younger animals.

By referring back to table 1 a possible explanation of the results can be seen. Basal diet 1 is the poorest. There are several contributing causes. Although this diet contained 10% wheat germ, it also contained the 0.5% cod liver oil which was mixed into the diet. Just as 5% wheat germ was ineffective in preventing skeletal muscle degeneration in young animals when cod liver oil was in the diet, so 10% wheat germ was ineffective in preventing reproductive failure in older animals fed a diet containing cod liver oil. This is in contrast to the work of Shimotori et al. ('40), who found that 5 or 10% wheat germ prevented muscular dystrophy in guinea pigs fed a diet containing cod liver oil. Embree ('47) stated that experimental diets containing fresh or hydrogenated vegetable oils provide so much tocopherol that supplementation with further doses is superfluous; nevertheless basal diet 1 was low in vitamin E, even though it contained 10% corn oil. Emmerie and Engel ('43) agreed with Embree that corn oil is high in tocopherol but Fisher ('45) showed that only a small percentage of its tocopherol is in the alpha form, none in the beta form, and the majority in the gamma form. Hove and Harris ('47), working with rabbits, showed that the alpha form is 5 times as potent as the gamma form in curing the creatinuria associated with muscular dystrophy. Moreover, Dam ('44) showed that vitamin E deficiency symptoms developed in chicks (exudates and encephalomalacia) when fat was present in the diet but seldom in its absence. Thus the 10% corn oil in basal diet 1, far from supplying the guinea pig with the required amount of vitamin E, actually appears to have increased the apparent vitamin E deficiency, making basal diet 1 the poorest of all.

The change in percentage composition from basal diet 1 to basal diet 2 was very slight, the only significant factor

being the removal of the 10% corn oil. This may have improved the diet, as compared to basal diet 1, by lowering the requirement for vitamin E.

During the war it was impossible to obtain soybean oilmeal for inclusion in this diet. For this reason linseed oilmeal and skim milk powder were substituted. The fish meal was reduced, the wheat bran removed, oats substituted for barley, the bone char increased from 2 to 4%, the brewers' yeast increased and the wheat germ decreased from 10 to 5%. In spite of all these changes, basal diet 3 was no better than basal diet 2; in fact, the percentage of litters produced was actually lower than on basal diet 2. In the light of our present knowledge it is concluded that decreasing the wheat germ from 10 to 5% was the only significant change, resulting in a lower vitamin E content in the diet. Since the requirement remained the same, the result was greater reproductive failure.

The marked change in percentage of litters produced came as a result of changing basal diet 3 to basal diet 4. As can be seen from table 1, the only essential change was that the cod liver oil was removed from the diet and given directly to the animals. In addition to this, vitamin E was given every other day to the guinea pigs. The results of feeding basal diet 4 suggest the same conclusion that was arrived at in the bioassay test. Vitamin E again proved effective in counteracting the vitamin E deficiency produced by mixing cod liver oil with the diet. Unfortunately, basal diet 4 has not been fed without vitamin E as a supplement. It is possible that this diet supplies sufficient vitamin E without any further addition. The only diet containing no cod liver oil which has been fed without supplementary vitamin E is basal diet 6. As was pointed out earlier, it is deficient in vitamin E. The difference between basal diet 4 and basal diet 6 is the 5% wheat germ in the former. In the absence of cod liver oil this might easily supply enough vitamin E.

The percentage of litters produced on basal diet 5 for both primipara and multipara is lower than the corresponding value for basal diet 4. Since the primipara on basal diet 5 received 12 mg alpha-tocopherol every other day, it is unlikely that this difference is traceable to vitamin E. No explanation can be offered.

It is evident from a consideration of the diets without green feed that the development of skeletal muscle degeneration can be completely prevented in young growing animals by 6 mg alpha-tocopherol in corn oil every other day. This same dose given to mature animals greatly reduces the incidence of hemorrhage and abortion, thereby permitting the birth of at least one living young. No skeletal muscle degeneration was seen in these older animals. In contrast to this, Pappenheimer and Goettsch ('41) found muscular dystrophy in one animal 312 days of age. Meunier et al. ('47) have shown that whereas young rabbits died on a vitamin E-deficient diet (including cod liver oil), older rabbits (1.5 kg) survived. This suggests a difference in vitamin E requirement associated with age which may explain the absence of lesions in the present experiment.

We turn now to a consideration of the diets in which green feed was fed along with the basal ration. Here again it is evident that primipara fare better than multipara on the same diet. The fact that basal diets 4 and 5 when supplemented with green feed were entirely satisfactory for guinea pigs having their first litters is evidence that a diet has been found which falls little short of meeting the dietary requirements of this species. The fact that only 80 to 90% of the multipara produced living young is probably not out of line with the performance of other species in this respect.

The improved response to diets including green feed over the response to those without green feed, as far as multipara were concerned, is probably an indication that green feed supplies something in addition to vitamin E which is required for successful reproduction.

CONCLUSIONS

It is evident that:

1. Wheat germ cannot be relied upon to supply vitamin E in the diet of guinea pigs when cod liver oil is mixed with the basal diet.

2. Synthetic alpha-tocopherol prevents skeletal muscle degeneration in young growing animals.

3. In primipara 6 mg of alpha-tocopherol every other day permits the birth of living young.

4. In multipara 6 mg of alpha-tocopherol every other day greatly reduces the incidence of hemorrhage and abortion, thereby increasing the percentage of litters produced.

5. Green feed supplies a factor (not vitamin E) required for prevention of hemorrhage and abortion in multiparous guinea pigs.

LITERATURE CITED

DAM, H. 1944 Studies on vitamin E deficiency in chicks. J. Nutrition, *27:* 193.

EMBREE, N. 1947 Vitamin E in laboratory diets. Arch. Biochem., *13:* 299.

EMMERIE, A., AND C. ENGEL 1943 The tocopherol (vitamin E) content of foods and its chemical determination. Zschr. Vitaminforsch, *13:* 259. Through Nutrition Abs. and Rev., *15:* 262, 1945.

FISHER, G. S. 1945 Determination of gamma-tocopherol in vegetable oils. Ind. Eng. Chem., Anal. Ed., *17:* 224.

HOVE, E. L., AND P. L. HARRIS 1947 Relative activity of the tocopherols in curing muscular dystrophy in rabbits. J. Nutrition, *33:* 95.

MADSEN, L. L., C. M. MCCAY AND L. A. MAYNARD 1935 Synthetic diets for herbivora with special reference to the toxicity of cod liver oil. Cornell Univ. Agr. Exp. Sta. Mem. 178, June, p. 63.

MANNERING, G. J. 1949 Vitamin requirements of the guinea pig. Vitamins and Hormones, *7:* 201.

MEUNIER, P., A. VINET AND J. JOUANNETEAU 1947 Sur les actions antagonistes de la vitamine E et des huiles de foie de poissons sur la croissance du lapin. Bull. Soc. chim. biol., *29:* 507.

PAPPENHEIMER, A. M., AND M. GOETTSCH 1941 Death of embryos in guinea pigs on diets low in vitamin E. Proc. Soc. Exp. Biol. Med., *47:* 268.

SHIMOTORI, N., G. A. EMERSON AND H. M. EVANS 1939 The role of vitamin E in the prevention of muscular dystrophy in guinea pigs reared on synthetic rations. Science, *90:* 89.

———— 1940 The prevention of nutritional muscular dystrophy in guinea pigs with vitamin E. J. Nutrition, *19:* 547.